DOCTOR OF PHILOSOPHY

Molecular characterisation of the Rhynchosporium commune interaction with barley

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Award date:
2016

Molecular characterisation of the *Rhynchosporium commune* interaction with barley

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Thesis submitted for the degree of
Doctor of Philosophy

University of Dundee

September 2016

Contents

CONTENTS II

LIST OF FIGURES VII

LIST OF TABLES X

LIST OF ABBREVIATIONS XI

ACKNOWLEDGEMENTS XIV

DECLARATION XV

STATEMENT XV

ABSTRACT XVI

1. GENERAL INTRODUCTION 1

1.1 Economic importance of *R. commune* infection on barley 1
1.2 Introduction to *R. commune* 2
1.2.1 Origin and host specialisation of *R. commune* 2
1.2.2 Classification 4
1.2.3 Epidemiology 7
1.2.4 Infection process and disease symptoms 9
1.2.5 *R. commune* in the plant apoplast 12
1.3 Pathogen - Host Interactions 12
1.3.1Evolutionary basis of host-pathogen interactions 12
1.3.2 Effectors and their role in disease 17
1.3.3 *R. commune* effectors – Necrosis Inducing Peptides 19
1.3.4 *AvrRrs1 – Rrs1* interaction 22
1.4 Barley resistance to *R commune* 23
1.4.1Major *R* gene and partial resistance 23
1.4.2Molecules involved in a resistant response 24
1.5 Pathogen evasion of plant defence 25
1.6 Scope of this thesis 27

2. MATERIALS AND METHODS 29

2.1 Plant seedling growth 29
2.2 Culturing and storage of micro-organisms
2.2.1 Fungi 29
2.2.2 Bacteria 30

2.2.3 Harvesting of fungal spores 30
2.3. *R. commune* infection of barley 30
2.3.1. Detached leaf assay 30
2.3.2 Infection time course 31
2.4 Microscopy 32
2.4.1 Lacto phenol trypan blue staining 32
2.4.2 Sample preparation for confocal microscopy 32
2.4.3 Confocal laser scanning microscopy (CLSM) 32
2.5 Gene expression 33
2.5.1 Total RNA, mRNA extraction and cDNA synthesis 33
2.5.2 Real-time RT-PCR (qRT-PCR) 33
2.6 Cloning strategy and expression plasmids construction 34
2.6.1 Preparation of competent yeast cells 34
2.6.1 Preparation of *S. cerevisiae* competent cells 35
2.6.2 *P. pastoris* electro competent cells 35
2.6.3 Yeast re-combinational cloning (YRC) 35
2.6.4 Plasmids used for YRC 36
2.6.4.1 Cloning plasmid 36
2.6.4.2 Expression plasmids 36
2.7 Transformation protocols 36
2.7.1 *E. coli* transformation 36
2.7.2 *S. cerevisiae* transformation 37
2.7.3 *P. pastoris* transformation 38
2.7.4 Electroporation transformation of *R. commune* 38
2.7.5 Transformation of fungal protoplasts 39
2.7.5.1 Protoplast preparation 39
2.7.5.2 Protoplast Transformation 39
2.7.6 Screening of yeast transformants by colony PCR 41
2.7.7 Protein expression analysis in *P. pastoris* cultures 41
2.7.1 Protein concentration 41
2.7.2 Protein purification 41
2.8 Molecular biology protocols 42
2.8.1 Fungal & plant DNA extractions 42
2.8.2 Plasmid extraction 42
2.8.2.1 Plasmid extraction from *E. coli* 42
2.8.2.2 Plasmid extraction from yeast cells 42
2.8.2 Polymerase chain reaction 43
2.9 Proteomics protocols 44
2.9.1 Protein extraction 44
2.9.2 Protein visualisation 45
2.9.2.1 SDS-polyacrylamide-gel electrophoresis 45
2.9.1.2 Western blotting 45
2.9.3 Proteome analysis of barley apoplast 46
2.9.3.1 Apoplastic fluid extraction of barley leaves 46
2.9.3.2 Enzyme contamination assay 46
2.9.3.3 Sample preparation for MS analysis: In gel digestion 47
2.9.3.4 Sample preparation for MS analysis: In Solution Digestion 48
2.9.3.5 Sample cleaning using C18 Ziptip columns 48
2.9.3.7 Dimethyl labelling procedure 49

2.10 Polysaccharide binding assay 50
2.11 In Vitro Fungal Growth Assays 50
2.12 Bioinformatic analysis 51
2.12.1 Sequence analysis tools 51
2.12.2 Proteomic analysis 52
2.12.3 Genome databses 52

3 IDENTIFICATION AND CHARACTERISATION OF *R. COMMUNE* CANDIDATE EFFECTOR GENES 53

3.1 Introduction 53
3.2 Results 55
3.2.1 Background – Bioinformatics pipeline reveals a plethora of candidate *R. commune* effectors 55
3.2.2 Candidate effector sequence analysis 57
3.2.3 Homology of *R. commune* candidate effectors to other fungal proteins 57
3.2.4 *R. commune* candidate effector gene expression *in planta* and *in vitro* 60
3.2.4.1 Analysis of candidate effector expression in *R. commune* strain L2A during infection in planta 60
3.2.4.2 Analysis of expression of selected candidate effectors during infection *in planta* and culture media using two different *R. commune strains* 64
3.2.5 *R. commune* transformation to elucidate gene function 70
3.2.6 Analysis of the apoplastic proteome during infection reveals the presence of potential effectors 73
3.2.6.1 Enzyme contamination assay 73
3.2.6.2 *R. commune* proteins identified in the apoplast 74
3.3 Discussion 80

4 IDENTIFICATION AND CHARACTERISATION OF A FAMILY OF LYSM-DOMAIN PROTEINS 84

4.1 Introduction 84
4.2 Results 87
4.2.1 A large family of LysM domain genes identified in the *R. commune* genome 87
4.2.2 Phylogenetic analysis of LysM domains from members of *R. commune* LysM domain protein family 92
4.2.3 Expression analysis of genes coding for LysM domain proteins reveals upregulation during infection of barley 95
4.2.4 RcLysM3 protein shares sequence homology with Z. tritici Mg3LysM and *C. fulvum* Ecp6 102
4.2.5 In vitro analysis confirms the ability of RcLysM3 to bind chitin 103
4.2.6 RcLysM3 does not provide protection against plant hydrolytic enzymes 106
4.2.7 Correlation between virulence/avirulence of *R. commune* strains on barley cultivar La Mesita and a SNP change in the RcLysM3 allele 107
4.3 Discussion 109

5 ANALYSIS OF BARLEY RESISTANCE TO *R. COMMUNE* 114

5.1 Introduction 114
5.2 Results 117
5.2.1 *Rrs3*, *Rrs4* and *Rrs13* show a high level of resistance to *R. commune* isolates 117
5.2.2 Analysis of asymptomatic infection on barley lines using *R. commune* strain 214-GFP 119
5.2.3 Analysis of asymptomatic infection of barley landraces using *R. commune* strain 214-GFP 122
5.2.4 Quantitative proteomics 124
5.2.4.1 Confirmation of barley response to *R. commune* during infection 124
5.2.4.2 Sequence analysis of defence related proteins identified in barley apoplastic proteome 126
5.4.5.3 Analysis of protein expression reveals upregulation of (1-3) β-glucanase and α-L arabinofuranosidase in Atlas 46 at 4 dpi 129
5.3 Discussion 133

6 CHARACTERISATION OF *R. COMMUNE* INTERACTION WITH *N. BENTHAMIANA* *136*

6.1 Introduction 136
6.2 Results 139
6.2.1 Asymptomatic growth of *R. commune* does occur on the model plant species *N. benthamiana* 139
6.2.3 Growth of *R. commune* strain 214-GFP on non-plant surfaces 143
6.2.4 *R. commune* gene expression during growth on non-host *N benthamiana* *1*44
6.2.4.1 Analysis of NIP expression on non-host *N benthamiana* 144
6.2.4.2 Analysis of expression during growth on *N. benthamiana* leaves of the *R. commune* effector RcINS1 inducing cell death in *N. benthamiana*. 147
6.3 Discussion 148

7 GENERAL DISCUSSION 151

8 REFERENCES 158

9 APPENDIX 181

9.1 Liquid and agar media 181
9.1.2 CZV8CM agar 181
9.1.2 Potato Dextrose Broth / Agar (PDB /PDA) 181
9.1.3 Yeast Peptone Dextrose (YPD) 181
9.1.4 YNB + glucose 182
9.1.5 Luria Broth and Agar plates (LB) 182
9.1.6. Synthetic complete minus uracil (SC-Ura) 182
9.1.7 Low salt Luria broth (LB) 182
9.1.8 SOC media 183
9.2 Lacto phenol trypan blue solution 183
9.2.1 Staining solution 183

9.2.2. De-staining solution 184
9.3 Solutions and reagents for transformation 184
9.3.1 Protoplast preparation 184
9.3.1.1. KC solution 184
9.3.1.2. MT solution 184
9.3.1.3. KC-MT solution 184
9.3.1.4 Protoplasting enzymes 185
9.3.1.5 PEG solution 185
9.3.2 Yeast transformations 185
9.3.2.1 SORB solution 185
9.3.2.2 PEG/LiAc 185
9.3.2.3 TE buffer 186
9.3.2.4 STET buffer 186
9.4 Solutions and reagents for proteomic experiments 186
9.4.1 Protein extraction buffer 186
9.4.2 PBS buffer 187
9.4.3 Blocking solution 187
9.4.4 Bead elution buffer 187
9.4.5 HNT buffer 187
9.4.6 HN buffer 188
9.4.7 Trypsin solution 188
9.5 List of intensity values for plant proteins identified in the apoplastic fluid of infected and non-infected barley leaves at 4 and 7 dpi 189
9.6 List of proteins identified in the apoplastic fluid from cultivars Optic, Atlas and Atlas46, infected and non-infected leaf material 195

List of figures

Figure	Title	Page
1.1	Spores of *Rhynchosporium commune* and barley cultivar Optic infected with *R. commune*	6
1.2	Representation of the epidemiology of *hynchosporium commune*	8
1.3	Schematic diagram showing the progress of *R. commune* infection on its host barley	11
1.4	The 'ZigZag' model	16
1.5	*R. commune* infecting the plant apoplast.	21
3.1	Pie chart representing the percentage of highest level of transcript abundance of *R. commune* candidate effectors at different time points of infection *in planta*	61
3.2	Relative transcript abundance of *Rhynchosporium commune* candidate effectors during infection of susceptible barley cultivar Optic with *R. commune* strain L2A	62
3.3	Relative expression of low abundance transcript of *Rhynchosporium commune* candidate effectors during infection of susceptible barley cultivar Optic with R. commune strain L2A	63
3.4	Relative transcript abundance of *R. commune* candidate effector *Rc_2835* during infection of barley with *R. commune* strain L2A	63
3.5	Relative expression of selected *R. commune* genes during infection of susceptible barley cultivar Optic with *R. commune* strain L2A	67
3.6	Relative expression of selected genes during infection of susceptible barley cultivar Optic with *R. commune* strain 214-GFP	68
3.7	Confocal images of the infection progress following inoculation of susceptible barley cultivar Optic with *R. commune* strain 214-GFP	69
3.8	Genotyping of *Rhynchosporium commune* transformants.	72
3.9	Percentage of *Rhynchosporium commune* proteins identified in barley apoplastic fluid within each biological process category	79

List of figures continued

Figure	Title	Page
4.1	Schematic amino acid sequence diagrams of LysM domain proteins	88
4.2	PRALINE multiple alignment of all LysM domains identified within *Rhynchosporium commune* proteins against the LysM domains from *Cladosporium fulvum effector Ecp6 showing conserved amino acids*	93
4.3	Phylogenetic tree showing the relationship between the LysM domains from *Rhynchosporium commune* LysM domain proteins and *Cladosporium fulvum* Ecp6	94
4.4	Relative expression of genes coding for LysM domain proteins during infection of susceptible barley cultivar Optic with *Rhynchosporium commune* strain L2A	96
4.5:	Relative expression of genes coding for LysM domain proteins during infection of barley cultivar Optic with *R. commune* strain 214-GFP	97
4.6	Relative expression of LysM genes during growth in PDB media using *Rhynchosporium commune* strain L2A	100
4.7	Relative expression of LysM genes in *Rhynchosporium commune* strain 214-GFP during its growth in PDB media	101
4.8	Alignment of full length protein sequences of RcLysM3, Mg3LysM and CfEcp6	103
4.9	3D predicted image produced by CHIMERA of RcLysM3	105
4.10	Ruby stained protein gel showing RcLysM3-V5 protein co-precipitating in the pellet (P) of chitin and chitosan, but only present in the supernatant (S) of cellulose and xylan.	105
5.1	Confocal LASER microscopy images of *Rhynchosporium commune* strain 214-GFP infection CI11549, BC Line 30 & Optic	120
5.2	Confocal LASER microscopy images of *Rhynchosporium commune* strain 214-GFP infection on SLB 66-024 and SLB 67-015	123
5.3	Confocal LASER microscopy images of *Rhynchosporium commune* strain 214-GFP infection at 4 dpi and 7 dpi on susceptible Optic (A & B), partially resistant Atlas (C & D) and resistant Atlas 46 (E & F)	125
5.4	Proteins highly abundant at 4 dpi. Intensity values in apoplast samples	131
5.5	Proteins highly abundant at 7 dpi. Intensity values in apoplast samples	132

List of figures continued

Figure	Title	Page
6.1	Confocal microscopy images of *Rhynchosporium commune* strain 214-GFP on *N. benthamiana* line CB173 expressing red plasma membrane at 5 & 9dpi	140
6.2	Confocal microscopy images of *Rhynchosporium commune* strain 214-GFP on *N. benthamiana* line CB173 expressing red plasma membrane at 15 dpi	141
6.3	*R. commune* strain 214-GFP on *Nicotiana benthamiana* line CB173 expressing red plasma membrane at 28 dpi & *N. benthamiana* plants inoculated with *R. commune*	142
6.4	Confocal image of *Rhynchosporium commune* strain 214-GFP spores on different surfaces	144
6.5	Relative expression of NIPs in *Rhynchosporium commune* strain 214-GFP during its growth on *N. benthamiana*.	146
6.6	Relative expression of *RcINS1* in *Rhynchosporium commune* strain L2A during infection on barley	147
6.7	Relative expression of *RcINS1* in *Rhynchosporium commune* strain 214-GFP during its growth on *N. benthamiana*	148

List of tables

Table	Title	Page
2.1	PCR conditions	44
3.1	Sequence analysis of *R. commune* candidate effectors and homology to other fungal proteins	59
3.2	Determination of the contamination of apoplastic extracts	74
3.3	SignalP and localisation predictions of *R. commune* proteins identified in apoplastic fluid	77
3.3	Intensity of identified apoplastic proteins, BLASTp hit and predicted biological function	78
4.1	Amino acid sequence analysis of *Rhynchosporium commune* LysM domain proteins identified in *R. commune*	91
4.2	Correlation of the Gln (E) and Glu (Q) allele with the virulence and avirulence of *Rhynchosporium commune* isolates on barley cultivar La Mesita	108
5.1	Virulence testing results of barley lines containing Rrs3 (Abyssinian), Rrs4 (Cl11549) & Rrs13 (BC Line 30) resistance genes inoculated with *Rhynchosporium commune* strains L32B, L43D, L73A, L77, UK7, AU2 & 214-GFP, at 21 days post inoculation	118
5.2	Sequence analysis of potential defence related proteins identified in the barley apoplast	128

List of abbreviations

AVR	Avirulence
BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
CBD	Chitin binding domain
cDNA	Coding DNA
CRISPR	Clustered regularly interspaced short palindromic repeats
CWDE	Cell wall degrading enzymes
DAMP	Damage Associated Molecular Pattern
DMSO	Dimethly sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EB	Elution Buffer
ETD	Effector triggered defence
ETI	Effector Triggered Immunity
ETS	Effector Triggered Susceptibility
F	Forward
GFP	Green Fluorescent Protein
Gln	Glutamine
Glu	Glutamate acid
HR	Hypersensitive Response
Hyg	Hygromycin
kDa	Kilo dalton
LB	Luria Bertani
mRNA	Messenger Ribonucleic acid
MAMP	Microbe Associated Molecular Pattern

List of abbreviations (continued)

MS	Mass spectrometery
NCBI	National Center for Biotechnology Information
NIP	Necrosis inducing peptide
NHR	Non host resistnce
OD	Optical Density
ORF	Open reading frame
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PR	Pathogensis related
PRR	Pattern Recognition Receptor
PTI	PAMP Triggered immunity
R	Resistance
R gene	Resistance gene
Rev	Reverse
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT-(q)PCR	Quantitative Real Time PCR
SDW	Sterile distilled water
SDS	Sodium Dodecyl Sulfate

List of abbreviations (continued)

SNP	Single nucleotide polymorphisms
TBS	Tris Buffered Saline
TM	Transmembrane domain
UTR	Untranslated region
YPD	Yeast peptone dextrose
YRC	Yeast recombinational cloning

Acknowledgements

I would like to dedicate this to my daughter Elana, thank you for your patience and tolerating a mum who has had her head firmly stuck in the world of plant pathology for the past four years! I hope, that through the completion of my thesis you will realise that anything in life is possible with a bit of determination and perseverance and in the future, inspires you to follow your dreams.

To my partner Billy, who has always been there to support and encourage me. I am grateful of all the times you have given me a shoulder to cry on, the positive words and motivation when I wanted to give up.

Lastly, I wish to dedicate this work in memory of my parents; my mum Myra who passed on her thirst for knowledge and the ways which, throughout my life encouraged me to achieve, make a contribution to this world and always believed in me and; My dad, Gerry who showed me that hard work is at the root of all success and not to be afraid of getting my hands dirty! I know in my heart you would both be proud.

I would like to express a big thank all my supervisors, Anna Avrova, Paul Birch and Adrian Newton for the help over the past few years for guidance and support. I also would like to thank the HGCA and the JHI for funding my project. I would also like to thank Elli Gilroy who has been a kind mentor throughout my PhD and also gave me advice for thesis writing.

Completing this work would have been all the more difficult without the support and help of my work colleagues at the JHI – I would like to thank them all individually but there is too many to mention. I am grateful for the opportunity to meet a wonderful group of people and I am thankful of the friendships I have made.

Declaration

This thesis is my own composition. The results presented here are of investigations conducted by myself. Work other than my own is clearly indicated with references to relevant researchers and/or their publications. This work has not, in whole or in part, been previously presented for a higher degree.

The research was carried out at The James Hutton Institute, Invergowrie, Scotland, under the supervision of Dr Anna Avrova.

Statement

I certify that Louise Gamble, a candidate for the degree of Doctor of Philosophy in the University of Dundee, has fulfilled the relevant Ordinance and Regulations of the University Court, and is qualified to submit this thesis.

Dr A Avrova
Cell and Molecular Sciences,
The James Hutton Institute.

Prof. Paul Birch
Division of Plant Sciences,
University of Dundee.

Abstract

The molecular interaction between *Rhynchosporium commune* and its host barley was studied to gain a better understanding of the pathogen during infection and provide further characterisation of resistance in barley, using a combination of bioinformatics, transcript expression analysis, proteomics and confocal microscopy.

Expression analysis of potential effector sequences identified novel candidate effectors *Rc_10934*, *Rc_2091* and *Rc_2835* which showed the highest abundance during the biotrophic infection. A further two novel candidates *Rc07_03591* and *Rc07_02334* and a LysM containing protein (RcLysM3) were identified using a proteomic analysis of infected plant apoplast.

Further apoplastic analysis revealed some of the most abundant proteins that are present in *R. commune*'s infection toolkit. Cell wall degrading enzymes (CWDEs), virulence factors and proteins involved in detoxification were all highlighted as some of the main key players of pathogenesis.

A large family of LysM domain containing proteins was later identified in the *R. commune* genome. Expression profiling revealed the upregulation of some of the transcripts during infection, indicating a potential role in pathogenesis, whereas others were expressed *in vitro* indicating potential functions for the proteins in fungal growth and development. RcLysM3 containing 3 LysM domains and sharing similarities with the well-known *C. fulvum* Ecp6 effector was selected for further characterisation. Bioinformatics predictions showed a high affinity for chitin binding which was confirmed *in vitro*. Binding analysis revealed that it can also bind chitosan but not plant cell wall polysaccharides, indicating that it is potentially involved in the evasion of

plant immune responses. The presence of the effector was also identified in the apoplast of infected barley leaves using a proteomic approach.

R. commune strain expressing GFP was used to characterise differences in pathogen growth and colony morphology in response to different genetic backgrounds of barley using lines carrying the *Rrs3* (Abyssinian), *Rrs4* (CI11549) and Rrs13 (BC line 30) genes and barley landraces with uncharacterised resistance.

Rrs1 resistance was further analysed using comparative proteomics to identify proteins differentially expressed in resistant and susceptible cultivars. Pathogenesis related proteins - chitinase, glucanase and thaumatin-like protease, were identified in the barley apoplastic fluid and were shown to be upregulated during infection. In addition, serine carboxypeptidase and purple acid phosphatase proteins were identified that were novel to the barley resistance interaction but have been identified in other incompatible interactions as defence related proteins.

The final chapter of this thesis is dedicated to the analysis of asymptomatic growth of *R. commune* on the model dicotyledonous plant *N. benthamiana* and analysis of effector transcription during growth on a non-host. *R. commune* growth was shown to be confined to the leaf surface, with no evidence of plant cell deterioration in transgenic *N. benthamiana* plants expressing an mRFP-tagged plasma membrane protein. This system could be used for further research into non-host interactions and provides insights into the growth of *R. commune* on alternative plant species.

Chapter 1

1. General Introduction

1.1. Economic importance of *R. commune* infection on barley

Fungal plant pathogens represent a group of agronomically important microorganisms causing devastating diseases on some of the most important world crops. Among these pathogens, the fungus *Rhynchosporium commune* - causes one of the most damaging diseases of *Hordeum vulgare* (barley) worldwide.

Barley was one of the first cultivated grains and is a major food source for developing countries, known for its nutritional value and versatility. In addition, early maturation coupled with a high level of adaptability to stressful conditions allows it to grow in a wide variety of environmental conditions (Saisho & Takeda, 2011). Worldwide, barley production amounted to just under 150 million metric tonnes in 2015/2016 (http://www.statista.com/statistics/271973/world-barley-production-since-2008/).

Barley crop infected with *R. commune* drastically affects crop yields and seed produced will be of lower quality. Across the globe there can be losses averaging 10% due to pathogen infection (Zhan *et al*., 2008). In the United Kingdom, around two thirds of the barley crop are used for animal feed and the remainder of barley is mostly used in the malting and brewing distilleries (Newton *et al*., 2011; Newman & Newman, 2006). Yield loss associated with the presence of this disease equates to £7.2 million a year, despite treatment (HGCA, 2013).

A relatively high genetic variation rate is a characteristic of this pathogen which has enabled it to overcome resistance genes deployed in attempts to control it (McDermott

et al., 1988). However, utilising resistant cultivars is one of the most economically and environmentally beneficial methods for controlling the disease, providing a low input, cost effective strategy that can be used in combination with other control methods as part of an integrated disease management approach. There is a need to develop more effective and sustainable resistance to this pathogen and a deeper understanding of the molecular basis of host-pathogen interactions is a prerequisite for this.

1.2 Introduction to *R. commune*

1.2.1 Origin and host specialisation of *R. commune*

In accordance with the domestication hypothesis the origin of a plant pathogen is thought to have arisen from the geographical location of their modern host, co-evolving during the hosts' domestication (Zaffarano *et al*., 2006). It is generally assumed that at the centre of origin the degree of pathogen genetic diversity is high. The Fertile Crescent- a region in the middle east, gave rise to the wild progenitors of many important crops and subsequently the domestication of these crops such as barley (Badr *et al*., 2000; Zohary and Hopf, 1993). It is therefore a reasonable assumption that the origin of *R. commune* would be that of the Fertile Crescent; however studies over the years have suggested otherwise.

Characterisation of the genetic structure of field populations worldwide using Restriction fragment length polymorphisms (RFLPs), mating type frequencies and DNA fingerprinting showed that Scandinavian populations displayed higher allele richness in comparison to samples from all other areas (Zaffarano *et al*., 2006). Another study conducted by the same authors proposed that *R. commune* had emerged more recently,

approximately 1200-3600 years ago due to a host shift occurring in northern Europe (Zaffarano *et al*., 2008). Additionally, reconstruction of the genetic history of *R. commune* through the analysis of migration patterns showed that the emergence of this pathogen was in Northern Europe approximately 2500-500 years ago and after the domestication of barley. After establishment on its host it eventually migrated on infected seed reaching the Fertile Crescent (Brunner *et al.*, 2007). Subsequently, the evidence suggests that *R. commune* is of Scandinavian origin.

The fungus was first isolated from rye more than 100 years ago (Oudemans, 1987) and originally the genus contained only two accepted species - *R. secalis* and *R. orthosporium* (Goodwin *et al*., 2002). *R. commune* has been previously referred to as *R. secalis*, infecting host species barley, rye, triticale and other grasses. Comparative analysis of rye and barley *R. secalis* isolates resulted in the identification of two specialised forms developing only on the original host (Lededeva & Tvaruzek, 2006). Phylogenetic analysis and pathogenicity confirmation studies of *R. secalis* isolates revealed the emergence of 3 distinct species based on their host specialisation: *R. secalis* which infects rye and triticale, *R. agropyri* which infects *Agropyron* spp and *R. commune* infecting barley, other *Hordeum* spp and *B. diandrus* (Zaffarano *et al*., 2011). However, a recent paper has revealed that isolates of *R. commune* are pathogenic on Italian ryegrass (King *et al*., 2012).

R. commune is found in all regions of barley production of the world, with cool, semi-humid weather conditions favouring the disease. In the United Kingdom it occurs more frequently and aggressively in areas such as the north and south west due to the favourable growth conditions and is currently controlled by the use of resistant cultivars

and application of mixtures of fungicides with different modes of action (Zhan *et al*., 2008)

1.2.2 Classification

R. commune is a haploid fungus within the phylum known as Ascomycota- the largest phylum within the fungal kingdom, with around 65,000 described species (Kirk *et al*., 2008). However further classification for this pathogen has been difficult due to a deficiency of morphological features (Goodwin *et al*., 2002). Hence, *R. commune* is placed under *Incertae sedis* (Latin for 'of uncertain placement') for both order and family.

Ascomycetes reproduce sexually through the formation of ascospores, but no teleomorph (sexual reproductive stage) has been identified for *R. commune*. It has been suggested that *R. commune* may have lost the ability to undergo sexual reproduction (Foster & Fitt, 2004). However, populations of *R. commune* exhibit a high level of genetic diversity, with global total genetic diversity of field populations estimated at 74% and a 40% total genetic diversity found within a $1m^2$ field plot. (Salamati *et al*., 2000; Zaffarano *et al*., 2006; Zaffarano *et al*., 2008). Sexual reproduction is thought to generate a greater genetic diversity than asexuality (McDonald & Linde, 2002) therefore, the level of genetic diversity within *R. commune* populations implies that the fungus reproduces sexually. In addition there is other evidence to suggest that a teleomorph may exist. Both MAT1-1 and MAT1-2 idiomorphs (mating type genes) have been identified and an equal frequency of isolates belonging to opposite mating types has been observed in most populations (Linde *et al*., 2003). The characterisation of the MAT idiomorphs (Foster & Fitt, 2004) and molecular analysis of fungal

ribosomal internal transcribed regions (Goodwin *et al*., 2002) from *R. commune* suggests that the teleomorph, if in existence is closely related to the sexual stage of the fungi- *Pyrenopeziza brassicae* (causing leaf spot on oil seed rape and mustard plants) and *Oculimacula yallundae* (causing eyespot on wheat) (Foster & Fitt, 2004).

Ascomycetes reproduce asexually via the production of conidia, which are formed by mitosis at the tips of conidiophores (Reece *et al*., 2015). The spores of *R. commune* are beak-shaped, mono-septate and are produced directly from the hyphae as opposed to being formed by usual structures such as conidiophores. Small protuberances from the hyphal cells are produced and upon maturity separate from the cells to form spores (Brooks, 1928) (Figure 1.1 C & D).

The pathogen has been described as a hemibiotroph due to a long asymptomatic phase during infection. Hemibiotrophs can be defined as having a biphasic lifestyle, which involves an asymptomatic biotrophic phase followed by necrosis of host cells, caused by the secretion of degradative enzymes and cell death elicitors (Kelley *et al*., 2010). Similar to *R. commune*, plant infection by the hemibiotrophic pathogens from *Phytophthora* and *Colletotrichum* species leads to the death of surrounding host tissue at the later stages of infection (Hammond-Kosack & Jones., 1997).

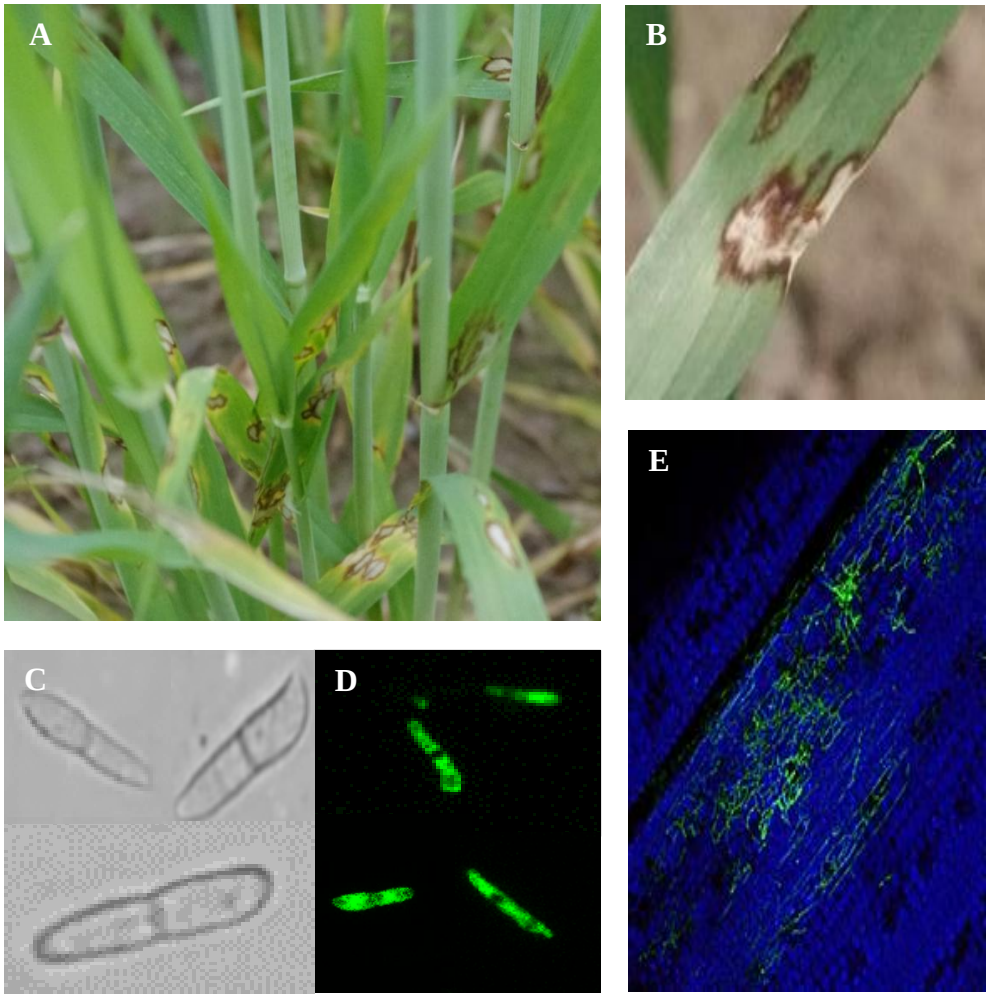


Figure 1.1: Spores of *Rhynchosporium commune* and barley cultivar Optic infected with *R. commune* A) Typical field symptoms of *R. commune* infection. B) Close up of an infected leaf showing scald like lesion. C) Light microscope image of *R. commune* conidia. D) Confocal microscope image of *R. commune* spores from GFP transformed isolate 214. E) Confocal image of the colonisation of susceptible barley cultivar Optic by *R. commune* strain 214-GFP at 10 days post inoculation. Chloroplast autofluorescence is false–coloured blue- emission range of 650-700 nm, while the 214-GFP hyphae are green - excitation of 488 nm and emission collection of 500-530 nm.

1.2.3 Epidemiology

The introduction of *R. commune* into new areas can be caused by seed-borne infection which most likely plays a role in long-distance dispersal (Fountaine *et al.*, 2010; Shipton *et al*., 1974) (Figure 1.2). The occurrence of symptomless infection in the barley plant contributes to spread of this disease via human mediated seed transport and may lead to the introduction of diverse races into new areas (Lee *et al*., 2002). After the first lesions appear and develop, dispersal of conidia (asexual spores) from infected leaves via splash dispersal can act as a source of secondary inoculum (Fitt *et al*., 1989) with the dispersal of conidia shown to be correlated with increases in rainfall intensity (Fitt *et al*., 1986). Primary inoculum can also originate from infection on crop debris and the pathogen has been shown to survive over winter on stubble debris but in the open field or buried in soil, the pathogen fails to survive during summer (Ayesu-Offei & Carter, 1971). There has been no alternative or secondary host identified during this stage of the lifecycle.

Rhynchosporium is a polycyclic disease resulting in the production of high levels of inoculum when conditions are favourable for the pathogen. The ability of *R. commune* to survive on crop debris results in a reservoir of inoculum that can splash- disperse throughout the growing season (Fitt *et al*., 1988). Airborne spores carried within small rain droplets have also been identified in field experiments acting as a source of inoculum which may also contribute to long distance dispersal (Fitt *et al*., 1987; Steadman, 1980).

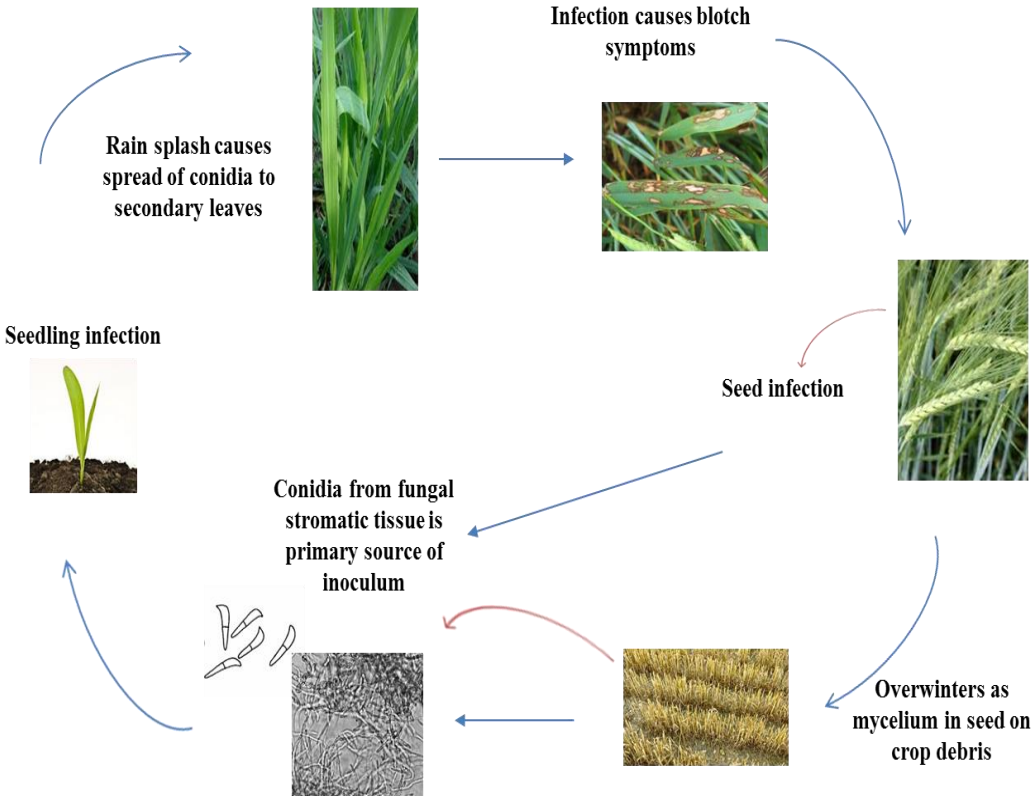


Figure 1.2: Representation of the epidemiology of *R. commune* adapted from HGCA, 2013. Infected seed and crop debris are sources of primary inoculum, infecting barley seedlings. The conidia are spread to secondary leaves via rain splash. Infection of *R. commune* causes lesion development on leaves of the barley plant.

1.2.4 Infection process and disease symptoms

Upon contact and after successful adhesion to the leaf surface, conidial germination occurs. Environmental factors play a major role in the ability of the pathogen to infect its host (Parker & Gilbert., 2004). Relatively low temperatures, leaf wetness and low light intensities have all been shown to be the optimum conditions for germination and germ tube elongation to occur in the *R. commune* infection process (Ryan & Clarke, 1975; Davis & Fitt, 1994). Cytological studies have observed the occurrence of germination at 12 hours post inoculation (Ayesu-Offei & Clare, 1970). In order to gain entry into the host tissue the pathogen must be able to overcome the initial physical barrier of the cuticle. Direct penetration through the cuticle has been observed in many studies of *R. commune* infection (Jones & Ayres, 1974; Jorgensen *et al*., 1993; Linsell *et al.,* 2011; Thirgnanasbanadam *et al*., 2011).

Similar to many other fungal pathogens *R. commune* develops appressorial structures, identified by Caldwell (1937) and described as small rounded structures found terminally on germ tubes. The enlarged areas at the tips of hyphae function to aid the pathogens' entry into the host (Figure 1.3 A). Following penetration, hyphae proliferate within the cellulosic region along the junction between the epidermal cell walls, remaining in the extracellular space (Figure 1.1 E and Figure 1.3 B). In contrast, biotrophic and other hemi-biotrophic pathogens are mainly intracellular, supress cell death to acquire nutrients from living host tissue and produce haustoria (feeding structures) to gain access to nutrients (Catanzaritic *et al*., 2006; Catanzaritic *et al*., 2007; Petre & Kamoun, 2014; Selin *et al*., 2016). It is thought that *R. commune* obtains nutrients from the degradation of the pectin-rich layer of the middle lamella by the production of pectinase as no haustoria (feeding structures) have ever been observed

(Jones & Ayres, 1974). Septate hyphae are arranged under the cuticle, surrounding the epidermal cells (Brookes, 1928) and the formation of an extensive mycelial network leads to the collapse of the cells resulting in the appearance of water soaked lesions on the surface of an infected leaf (Jones & Ayres, 1974) (Fig 1.3 C). Infection causes increased permeability of cell membranes which is the likely reason for the collapse and eventual death of the cells (Ayres, 1972). Collapse of the mesophyll cells occurs as the infection progresses (Fig 1.3 D) and lesions develop their characteristic necrotic irregular shaped, scald-like form (Figure 1.1 A & B). At this stage of the infection the pathogen is described as a necrotroph.

To obtain nutrients, necrotrophs are able to initiate plant cell death, secreting phytotoxic metabolites and producing reactive oxygen species. (Horbach *et al*., 2011; Wang *et al*., 2014). For *R. commune*, visible necrotic lesions occur during infection due to cell collapse and are characteristic of compatible reaction between a virulent pathogen and susceptible host. At this later necrotic stage, the front of the infection seems to move away from the necrotic cell suggesting that nutrient acquisition from dead or decaying matter may not be essential for this fungus (Avrova & Knogge, 2012) and therefore the fungus may not conform to the feeding mechanisms which are characteristic of a truly defined necrotroph. Upon maturity, the scald like lesions contain a grey centre surrounded by a dark brown margin (Figure 1.1 A & B) signifying the end of the infection cycle when sporulation occurs (Brookes, 1928).

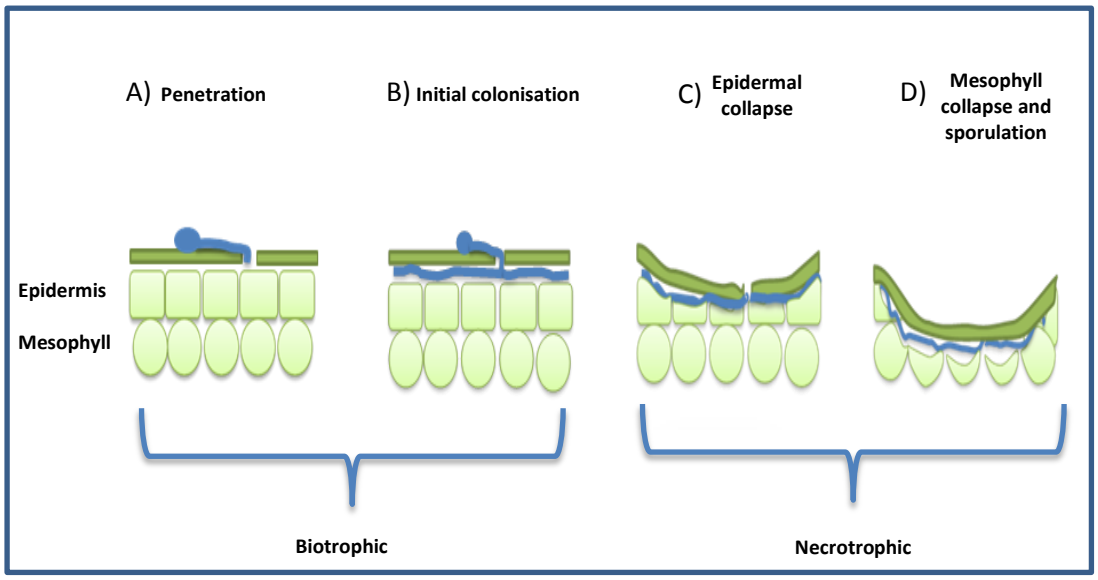


Figure 1.3 Schematic diagram showing the progress of *R. commune* infection on its host barley. A) Germ tube forms from the conidia and directly penetrates the leaf cuticle (24-48 hours). B) The fungus produces mycelium which grows between the cells, under the cuticle (2dpi – 8dpi). C) As the infection progresses the epidermal cells of the plant collapse (9dpi – 14dpi). D) Closer to the end of infection the mesophyll layer collapses (14dpi – 21dpi) and the pathogen sporulation occurs (~21dpi).

1.2.5 *R. commune* in the plant apoplast

Throughout the infection process, *R. commune* is confined to the plant apoplastic space. The survival of *R. commune* in the apoplast can be defined as its ability in the adaptation to metabolise available nutrients, tolerate preformed defence molecules and express pathogenicity and virulence factors that modulate host defences and metabolism (Doehlemann & Hemetsberger, 2013). The plant apoplast it an important compartment consisting of all the components outside of the plant cell membrane. It is composed of a series of gas filled channels in which the diffusion of air and liquid occur throughout the plant. Thus, it is fundamental to plants as it plays a major role in inter and intracellular signalling and transport of both water and nutrients (Sattelmacher, 2001). This dynamic and intriguing compartment is an understudied area of plant pathogen interactions. It is within the apoplast that secreted proteins, derived from both host and pathogen first interact and therefore determine the fate of an interaction (Gupta *et al*., 2015).

1.3 Pathogen - Host Interactions

1.3.1 Evolutionary basis of host-pathogen interactions

In an environment where exposure to numerous potential pathogens is inevitable, plants have developed a complex and multi-layered immune system to protect them from attack (Jones & Dangl, 2006; Dodds & Rathjen, 2010). From his research on the inheritance of pathogenicity of *Melamspora lini*, Harold Flor (1971) devised the now widely recognised gene-for-gene concept that describes the genetic interactions between pathogens and their hosts. Central to this, is that for every incompatible reaction (where disease does not occur), there is a resistance gene in the host and a corresponding *Avr*

gene in the pathogen. When either of these two is absent or not expressed, a compatible reaction (disease) occurs. Since the discovery of this concept there has been many host-pathogen systems discovered that conform to this description of genetic interactions (Van den Ackerveken *et al.,* 1992; Silué *et al.,* 1992; Jia *et al*., 2000; Ellis *et al*., 2007). The *R. commune* - barley pathosystem has been shown to follow the gene-for- gene hypothesis, in fact the *R. commune* – barley pathosystem was one of the first host-pathogen interactions identified to conform to the theory (Rohe *et al*., 1995). Successful pathogens have evolved some amazing strategies to manipulate plant defences through the deployment of molecules that aid in pathogenesis (termed effectors). Natural selection drives both pathogens and plants to evolve new strategies to overcome the obstacles presented by each other (Parker &Gilbert, 2004). This has resulted in a co-evolutionary arms race, described in its simplest form by the zig-zag model presented by Jones & Dangl in 2006.

In more detail, the zig-zag model proposes the first layer of plant bipartite defence is activated upon recognition of pathogen or microbial associated molecular patterns (P/MAMPs) which are broadly conserved among species or genera, such as bacterial flagellin and chitin from fungal cell walls (Felix *et al*., 1999; Kaku *et al*., 2006; Boller & Felix, 2009). The patterns are detected by pattern recognition receptors (PRR's) leading to the activation of PAMP Triggered Immunity (PTI) which can prevent the pathogen from infecting and colonising host tissues (Macho & Zipfel, 2014). Pathogens deploy effector proteins, which are generally lineage specific (Guyon et al., 2014) to overcome this first layer of defence (Stergiopoulos & De Wit, 2009) The second mechanism of plant immunity involves the direct or indirect recognition of pathogen effector molecules by polymorphic resistance proteins which are the products of major resistance genes. This layer of immunity is known as Effector Triggered Immunity

(ETI) and is essentially a more amplified and accelerated PTI response which often culminates in a Hypersensitive Response (HR) (Dangl & Jones, 2001; 2014; Hurley *et al*., 2014).

However, effector proteins associated with ETI have been largely associated with effector molecules from haustorial pathogens where recognition occurs in the cell cytoplasm (Lo Presti *et al*., 2015). For apoplastic pathogens it has been suggested that pathogen effectors are recognised at the cell surface (Stotz *et al*., 2014). At present, this theory would comply with effectors from *R. commune* as there has been no evidence to suggest they translocate into the host cell. In addition, immunity against apoplastic pathogens tends to be slower and isn't accompanied by a rapid cell death (Stotz *et al*., 2014). Therefore, the term Effector Triggered Defence (ETD) has been proposed (Figure 1.4). In addition, some effectors display wide distribution and elicit defence responses resembling typical MAMPS/PAMPs. LysM effectors for example, widely occur in the fungal kingdom and function to scavenge chitin oligosaccharides to prevent chitin induced plant defences (Bolton *et al*., 2008; de Jonge and Thomma, 2009) Indeed, several identified LysM effectors are fundamental to the lifestyle of fungal pathogens upon colonisation of their hosts (Marshall *et al*., 2011; Mentlak *et al*., 2012). Nep1 like proteins, best known for their cytotoxicity in dicot plants have been identified in fungi, bacteria, oomycetes and were shown to act as MAMPs (Oome *et al*., 2014). Furthermore, some PAMPs are narrowly conserved and several well-known PAMPs including LPS and bacterial flagellin also have virulence functions (Thomma *et al*., 2011).

Despite the substantial progress that has been made in the recent years based on the zig zag model for understanding how plants and fungal pathogens interact and co-evolve, more recent research and discoveries imply a continuum between the pathogen and

plant molecules involved in PTI and ETI rather than a complete line of distinction and argue that plant immune receptors recognising appropriate ligands, are the driving determinant of plant resistance; the amplitude of which is governed by the level required for plant immunity to be effective (Thomma *et al*., 2011).

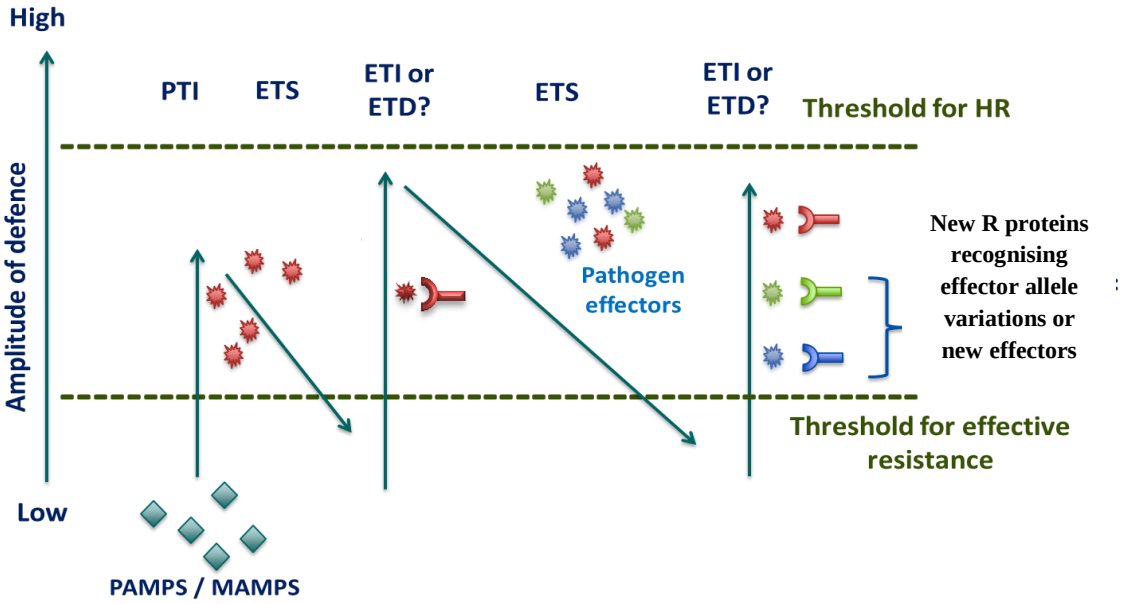


Figure 1.4: **The 'ZigZag' model adjusted from Jones & Dangl 2006 showing the evolution of pathogen-host interactions. Pathogen/molecular associated molecular patterns (PAMPs/MAMPS) are recognised by plants first layer of defence triggering PAMPs triggered immunity (PTI). Pathogen effector molecules are secreted to interfere with the plant defence. Plant resistance proteins recognise the effector molecules and mount the second layer of immunity termed Effector triggered defense (ETD). In comparison to other pathosystems a resistant interaction between *R. commune* and barley does not culminate in a cell death response (HR – hypersensitive response). Pathogen effectors evolve to prevent recognition and susceptibility occurs. In turn, plants evolve new resistance proteins to recognise modified or new pathogen effector molecules and the evolutionary cycle continues.**

1.3.2 Effectors and their role in disease

Many of the effectors that have been identified to date are typically secreted proteins that are often host specific, induced upon host colonisation and highly up regulated during infection (Jonge *et al*., 2011; Zhu *et al*., 2013). Fungal effectors are generally cysteine rich proteins which can be extracellular - secreted into the apoplast or xylem of host plants, or may be cytoplasmic - translocated into the host cells (Stergiopoulos & de Wit, 2009). Effectors have also been identified that move from an infected host cell into neighbouring cells. For example, two proteins from the rice pathogen *Magnaporthe oryzae,* initially secreted into the rice cytoplasm were identified in uninvaded neighbouring plant cells, possibly preparing host cells for invasion (Khang *et al*., 2010).

The presence of the RXLR and the Y/F/WXC motifs in *Phytophthora* and Powdery mildew effectors respectively, have aided in the identification of new effectors (Bhattacharj *et al*., 2006; Whisson *et al*., 2007; Goddfrey *et al*., 2010). Unfortunately, no specific motifs have been identified in *R. commune* candidate effector sequences so far. Many studies have incorporated the use of genome analysis and prediction pipelines which are based on the selection of the common characteristics of effectors, this has resulted in the rapid identification of many putative effectors from some important plant pathogens (Vleeshouwers *et al*., 2008; Bowen *et al*., 2009; Dong *et al*., 2011; Saunders *et al.*, 2012).

Effectors are highly diverse, possessing different functions dependant on the type of pathogen and the mode of infection. For instance, necrotrophs require the death of host cells in order to acquire nutrients and effectors would not only be required to manipulate

the host's metabolome but also to initiate cell death. The necrotrophic effector ToxA from the wheat pathogen *Pyrenophora tritici-repentis* stimulates cell death through the interference with photosynthetic electron transport (Manning *et al.*, 2005). In contrast biotrophic and some hemibiotrophic effectors have been shown to suppress host defence processes (Abramovitch & Martin, 2004). Recently Wingham *et al.* (2015) identified BEC1019, a candidate effector from the obligate biotroph *B. graminis f. sp hordei* that was capable of suppressing the HR. The biotrophic maize pathogen *Ustilago maydis* secretes chorismate mutase (Cmu1) that interferes with the host metabolism via the shikimate pathway, preventing the flow of chorismate into the salicylic acid (SA, involved in plant defence against biotrophic pathogens) biosynthesis branch (Djamei *et al*., 2011). Interestingly, genes encoding this effector were found in many genomes of symbionts, biotrophic and several hemibiotrophic plant pathogens but only rarely in necrotrophic plant pathogens and saprophytes (Djamei *et al*., 2011). Another example of plant hormone signalling manipulation by phytopathogens to access nutrients and counteract defence responses is coronatine production by hemibiotrophic bacterial pathogen *Pseudomonas syringae* (Zheng *et al*., 2012). Coronatine is a toxin mimicking the plant hormone jasmonic acid (JA) and promoting opening of stomata for bacterial entry, bacterial growth in the apoplast, systemic susceptibility, and disease symptoms (Zheng *et al*., 2012). Coronatine has also been shown to suppress SA accumulation through the NAC transcription factors (TFs).

Interestingly, pathogens that have been classified as necrotrophs have also been shown to supress host resistance. Candidate effector gene (SSITL) was recently identified in the necrotrophic pathogen *Scleotinia sclerotiorum* which suppresses the JA/ethylene signal pathway (Zhu *et al*., 2013), but it is likely that some of these pathogens are actually hemibiotrophs, rather than true necrotrophs. Avoidance of the host recognition

system has been shown to be the function for effectors of the tomato pathogen *Cladosporium fulvum*. This fungus colonises the apoplastic space similar to *R. commune* and secretion of the CfEcp6 and CfAvr4 effectors aid in the evasion of host recognition through the binding of chitin molecules to prevent detection by plant pathogen recognition receptors and the protection against chitinases (van den Burg *et al*., 2006; de Jonge *et al*., 2010).

1.3.3 R. commune effectors – Necrosis Inducing Peptides

Three necrosis inducing peptides have been characterised to date from the *R. commune* effector repertoire. The effectors are secreted from the fungal hyphae into the plant apoplast (Fig 1.5). The peptides were first identified in culture filtrates and in the leaves of a susceptible barley plant (Wevelsiep *et al*., 1991). The effectors have been studied greatly since their identification which has led to the discovery that two of these peptides may have a function in nutrient acquisition (Wevelsiep *et al*., 1991; Wevelsiep *et al*., 1993).

The *NIP1* gene codes for an 82 amino acid protein containing a 22 amino acid secretory signal peptide (Rohe *et al*., 1995). The mature NIP2 and NIP3 proteins are 93 and 98 amino acids respectively (Avrova & Knogge, 2012). All NIPs are cysteine rich containing 10, 6 and 8 cysteines respectively. A high proportion of cysteine residues indicates the protein's ability to survive in the apoplast. NIP1 has a conserved cysteine pattern and a hydrophobic domain (Gierlich *et al*., 1999). Cysteines are thought to be involved in the disulphide bridge formation, with globular proteins being more stable in the harsh environment of the apoplast.

NIP1 and NIP3 have been shown to stimulate the activity of barley plasmalemma H+ -ATPase by around 60% and are involved in symptom expression during infection (Wevelsiep *et al*., 1993). For NIP2 no function has been identified to date. A recent study revealed that the effector transcripts were highly abundant during the early stages of *R. commune* infection (Kirsten *et al*., 2012). Additionally, a rise in fungal biomass coincided with a sudden decrease in their expression suggesting that they are not associated with the later necrotrophic stages.

It is possible that the stimulation by NIP1 and NIP3 of the plasma membrane H+-ATPase, controlling the electrochemical gradient at the plant plasma membrane, may affect the regulation of essential membrane transport processes, such as nutrient export (Elmore & Coaker., 2011). Alternatively, this might help to release nutrients during the biotrophic stage of infection by creating an acidic environment in the apoplast creating optimal conditions for enzymatic degradation of the plant cell walls (Avrova & Knogge, 2012) or through the release of nutrients within the host cell. The potential role of apoplastic acidification has also been discussed for several other phytopathogenic fungi, including *Botrytis cinerea* and *S. sclerotiorum* (Prusky & Yakoby, 2003).

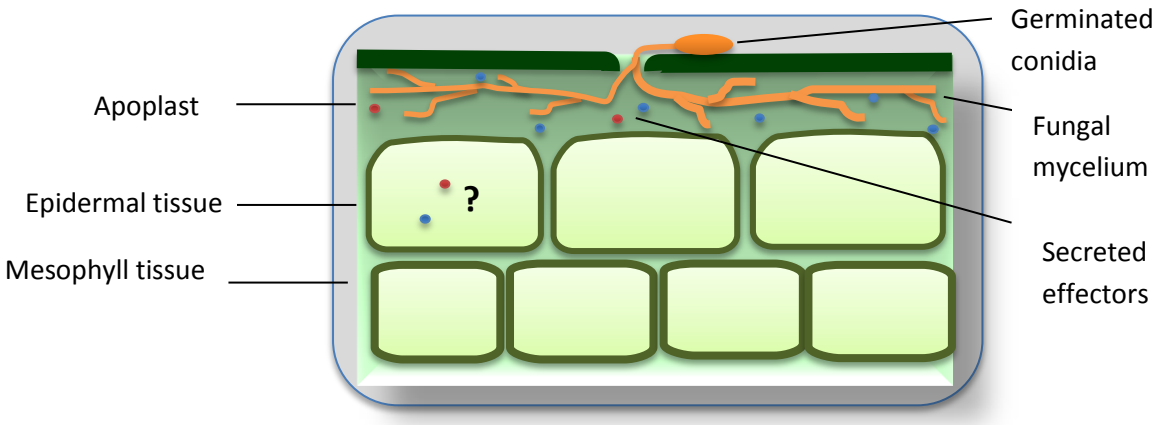


Figure 1.5: Diagram of *R. commune* infecting the plant apoplast. As the mycelium (orange) colonises the space around the epidermal tissues, effectors (blue and red) are secreted into the apoplast. No *R. commune* effectors have been shown to be translocated into the host cells, highlighted by the question mark in the plant host cell.

1.3.4 *AvrRrs1 – Rrs1* interaction

NIP1 (AvrRrs1) protein has been shown to elicit defence responses in cultivars carrying the cognate *Rrs1* gene (Hahn *et al*., 1993). Inoculation of an *Rrs1* barley cultivar with a race of *R. commune* carrying the *NIP1* gene resulted in a significant accumulation of the pathogenesis related protein PRHv-1 (Hahn *et al*., 1993). This plant response was observed preceding fungal penetration with NIP1 mRNA transcripts identified at very early infection time point. This is not surprising considering *NIP1* was shown to be highly up regulated at early time points of infection and therefore may be present already in the spores (Kirsten *et al*., 2012). This would account for the early activation of *Rrs1* mediated defence.

The products of major *R* genes can directly or indirectly recognise effectors encoded by the cognate *Avr* genes. The receptor ligand model has been proposed to describe the underlying mechanisms of the direct interaction (Ellis *et al.*, 2007). The implication of this model is that plants must carry large numbers of *R* proteins to enable them to have the ability of recognising many individual effectors. An indirect perception mechanism (the guard hypothesis) explains how multiple Avr proteins could be detected by a product of a single *R* gene (van der Biezan & Jones, 1998). This model suggests that the *Avr* gene products interact with the effector target modifying it in a way, allowing recognition by the R proteins.

While the actual perception mechanism of NIP1 by the resistance protein Rrs1 remains to be discovered, variants of the NIP1 protein that were inactive as Avr factors still possessed an efficient binding affinity to a single plasma membrane NIP1 receptor suggesting that the *Rrs1* gene does not encode the NIP1 receptor and the perception

mechanism is likely to follow the guard hypothesis (van' Slott *et al*., 2007). Additionally, a high deletion frequency rate of NIP1 has been identified (Shcurch *et al.*, 2004). The complete removal of an effector to evade *R*-mediated resistance is common of indirect interactions, whereas the direct interaction between an effector and the cognate R protein can be overcome more easily by mutations in the effectors that prohibit recognition (De Wit *et al.*, 2007; Jones & Dangl, 2006).

1.4 Barley resistance to *R commune*

1.4.1 Major *R* gene and partial resistance

Major *R* gene resistance or qualitative resistance which is host specific, recognises particular strains of a pathogen that express the corresponding *Avr* gene. (Lehnackers & Knogge, 1990; Zhan et al., 2007). In many cases, it can provide complete resistance to one or more strains of a pathogen. The resistance is usually controlled by a single dominant or semi dominant *R* gene (Kosack & Jones., 1997). A total of nine loci representing sixteen major *R* genes against *R. commune* have been mapped in the barley genome (Zhan et al., 2008). This includes the first major *R* gene discovered - *Rrs1*, representing a complex locus with either many tightly linked genes or multiple alleles, which has been mapped to chromosome 3H. The *Rrs2* and *Rrs12* loci have been located on 7H chromosome, *Rrs3* - on chromosome 4H, *Rrs13* - on chromosome 6H and 2 *Rrs15* loci on chromosome 2H and 7H (Zhan *et al*., 2008).

In contrast, quantitative resistance, also defined as partial resistance is often controlled by many genes that display a more continuous distribution and can be characterised by quantitative trait loci (QTL) (Zhan *et al*., 2008). The main characteristic of this

resistance is the reduction of pathogen infection. Partial resistance has been shown to be controlled by plant development and tends to increase during plant aging (Develey-Rivière & Galiana, 2007; Vergne *et al*., 2010).

1.4.2 Molecules involved in a resistant response

Previous studies have shown that resistance against *R. commune* results in the earlier accumulation of pathogenesis related proteins in tissues of resistant compared to susceptible barley plants. (Hahn *et al.,* 1993; Steiner-Lange *et al.,* 2003). High levels of transcripts encoding enzymes such as the Lipoxygenase gene (LoxA) which have been previously identified during pathogen induced defence responses were also detected in the epidermis of resistant plants (Steiner-Lange *et al*., 2003). Interestingly, *R. commune* has been shown to grow within barley tissue in resistant plants (Thirugnanasambandam *et al*., 2011). However, yield penalty associated with the growth of the pathogen in resistant cultivars is as yet unknown. Infection of a resistant cultivar with a GFP-expressing isolate revealed that pathogen was unable to form a fully functional mycelial network in comparison to growth on a susceptible cultivar. This would suggest that plant resistance is involved in the restriction of this fundamental stage of *R. commune* development during infection. In the barley – *R. commune* pathosystem pre and post penetration stages may be affected by partial resistance whereas major *R* gene mediated resistance may play an important role in post-penetration stages of pathogen development (Zhan *et al*., 2008). Additionally, host cell wall alterations in the form of appositions have been shown to be an induced plant immune mechanism resulting in the prevention of pre- penetration (Xi *et al*., 2000).

Commonly associated with major *R* gene resistance is the initiation of local host cell death to prevent further pathogen colonisation, termed the HR (Hypersensitive response). For *R. commune* – barley interactions no HR has been observed in cultivars with *Rrs1* resistance gene. Although there was evidence of small necrotic flecks, resembling HR in some resistant cultivars inoculated with certain isolates of *R. commune* (Bjornstad *et al*., 2002). The absence of HR is not exclusive to this pathosystem. For example, HR is not activated within the incompatible interaction between *C. fulvum* and host plant tomato (Hammond & Jones, 1994). Suggested by Morel (1997), a certain threshold level that activates a cell death response may need to be reached before the irreversible event of HR is initiated and the interaction of some pathogens and their hosts may not accumulate a high enough level for this activation. It has yet to be shown if a HR is associated with the resistance of all known *R* genes in barley plants as studies conducted so far with *R. commune* pathosystem have focused mainly on the *Rrs1* and *Rrs2* phenotypes.

1.5 Pathogen evasion of plant defences

Effectors that are recognised by the corresponding resistance gene in the host are subsequently a liability to the pathogen, negatively affecting the pathogens ability to infect its host. Pathogens have evolved diverse strategies to avoid plant recognition through the inactivation of avirulence genes. Several mechanisms including frameshift mutations, gene deletion and non-synonymous point mutations can result in the inactivation of an avirulence gene (Joosten *et al*., 1997; Na *et al*., 2013; Yin *et al*., 2013). The two latter mechanisms have been adopted by *R. commune* to evade *Rrs1* mediated recognition (Rohe *et al*., 1995). Amino acid alterations resulting from single

nucleotide polymorphisms were identified in the NIP1 protein affected elicitor activity (Fiegen and Knogge, 2003). A study conducted on a worldwide population of NIP1 isoforms showed a 45% deletion rate in comparison to NIP2 and NIP3 which were present in nearly all isolates (Schurch *et al*., 2004). Additionally, isolates that lacked the *NIP1* gene were also present in areas where isolates still carried *NIP1* gene, further indicating that the gene may be dispensable for the fungus. Many other pathogens have been shown to alter the structure of an avirulence gene to evade recognition (Stergiopoulos *et al*., 2007). The complete deletion of an effector gene could indicate a non-essential function. Thus, effector genes that are present in all isolates and show less variation are more likely to be required by the pathogen and not so easily disregarded. Subsequently, *R* genes recognising these *Avr* genes are likely to be more durable.

1.6 Scope of the thesis

Clearly marked in the literature review is the serious economic loss that *R. commune* causes to the barley industry. Despite that the fungus was isolated over 30 years ago, many important characteristics of this fungus are still relatively elusive. The main obstacle in controlling the pathogen is the identification of durable barley resistance. Achieving better control of this disease has been challenging due to the lack of understanding of the molecular mechanism of resistance to this pathogen and hence the limited knowledge of resistance proteins that govern defence mechanisms. Therefore, on the plant side, a deeper understanding of asymptomatic infection of *R. commune* on its host barley in combination with addressing the lack of knowledge of barley resistance is required. Furthermore, only few *R. commune* effectors have been reported to date. The identification and characterization of these effectors, however, is crucial to gain a better understanding of the infection process and will also facilitate the discovery of avirulence genes. The focus of the research is aimed at the identification of effectors that are essential for the pathogen and are less likely to be altered under selection pressure.

The overall aim of the research was to elucidate the molecular mechanisms of both pathogen infection and host resistance to aid in the discovery of more durable barley resistance to *R. commune*. To achieve this, candidate effector identification and characterisation was the starting point. Selection criteria and experimental outcomes enhanced the discovery of conserved candidate effectors. Plant resistance to *R. commune* was assessed using different barley *R* genes with asymptomatic infection being monitored to characterise potential resistant lines. Further insight into *Rrs1* barley resistance elucidated plant molecules involved in defence. Lastly, asymptomatic

growth on alternative plant species was touched upon, giving a better insight into the potential relationships of *R. commune* on alternative plant species.

Objectives

- Utilise genome and RNA sequence data to identify and select conserved candidate effectors through sequence analysis
- Analysis the expression profiles in planta and during vegetative growth
- Determine if candidates are essential for pathogenicity using targeted gene disruption
- Validate the presence of candidate effector protein in the apoplast during infection
- Functionally characterise selected effector candidates
- Microscopically analyse asymptomatic pathogen growth on different barley resistant resources
- Use quantitative proteomics to gain further insight into *Rrs1* resistance mechanism through the identification of plant molecules involved in a resistant response
- Analyse the asymptomatic growth of *R. commune* on an alternative plant species

CHAPTER 2

2. Materials and Methods

2.1 Plant growth

All plants used in this research were grown in compost from the JHI which contained 400 L sand, 2.5 kg dolomite limestone, 2.5 kg ground limestone 100 L perlite, 1.5 kg Synchrostart fast release Fertilizer, micronutrients (B, Cu, Fe, Mn, Mo, Zn), and Intercept insecticide. *Hordeum vulgare* plants were grown under glasshouse conditions at 19 ^{o}C with a 16-h day photoperiod for approximately 8-11 days. *Nicotiana benthamiana plants* were grown under glasshouse conditions at 22-24 °C with a 14-hour day photoperiod for 4-5 weeks.

2.2 Culturing and storage of micro organisms

2.2.1 Fungi

Rhynchosporium commune isolates from the culture collection at the James Hutton Institute were grown on CZV8CM agar medium (Newton, 1989) at 17 ^{o}C in the dark. Cultures were maintained by transferring of sporulating mycelium onto fresh CZV8CM agar plates every 2 weeks. Conidia stocks were made of each isolate and stored at -80 ^{o}C.

Trichoderma viride obtained from the Wageningen Universtiy, the Netherlands was cultured on Potato Dextrose Agar (PDA) for 14 days at 17 ^{o}C until spores were produced.

Saccharomyces cerevisiae strain FY834 and *Pichia pastoris* Strain GS115 were grown from glycerol stock stored at -80 ^{o}C on Yeast Extract Peptone Dextrose (YPD) media at 28 ^{o}C for 2-3 days.

2.2.2 Bacteria

Escherichia coli cells (MAX Efficiency® DH5α™ Competent Cells, Invitrogen) were grown overnight at 37 ^{o}C on Luria-Bertani (LB) agar medium with the addition of an appropriate antibiotic. All competent *E. coli* cells were stored at -80 ^{o}C in accordance with the manufacturer's instructions.

2.2.3 Harvesting of fungal spores

T. viridae spores and *R. commune* conidia were harvested from approximately 14-day-old cultures by scraping the mycelial mat with a spatula following the addition of 5 mL of sterile distilled water (SDW). The suspension was filtered through glass wool or a filter unit containing 30µm filter (Millipore™). The suspension was centrifuged for 3 min at 1600 g and washed with SDW. This step was repeated three times. Spore concentration was calculated using a haemocytometer.

2.3. *R. commune* infection of barley

2.3.1. Detached leaf assay

A 3-4 cm section from the first leaf (coleoptile) was cut and placed into rectangular boxes containing distilled water agar containing 120 mg/L benzimadazole. Using a haemocytometer, the concentration of *R. commune* spore suspension was adjusted to 1 x 10^5 spores/mL. Leaves were gently abraded with a small, fine haired brush and 10 µL

of spore suspension was pipetted onto the area. The fully susceptible cultivars Maris Mink or Optic were used as a control in each of the experiments to assess the pathogenicity of each isolate. SWD was used as a negative control to assess any mechanical damage caused by abrasion technique. Boxes containing the inoculated material were incubated in a controlled environment cabinet (Leec, model LT1201), light intensity of 200 µmol m^{-2} s^{-1} at 17 ^{o}C. Inspection of lesion formation began at 10 days post inoculation (dpi) and measurements continued until the leaf segment became too chlorotic to assess.

2.3.2 Infection time course

Approximately 200 seeds were planted and grown in a propagator at 17 ^{o}C for up to 7 days or until plants were around 5-10 cm tall. Spores were collected as previously described. The seedlings were spray inoculated with a spore suspension of 20 mL at a concentration of ~4 x 10^{7} with 1/1000 vol. of tween 20 (Sigma). To promote spore germination and the infection process the inoculated plants were kept in the dark for 24 hours and at high humidity for 48 hours. Plants remained in the propagator under high humidity conditions for the remainder of the experiment. Samples containing segments from 6 different leaves were collected over a period of 14 days representing important time points of infection: pre and post penetration stages, epidermal colonisation and necrotrophy. Additionally, samples were taken before plants were inoculated to act as a control. Samples were stored at -70 ^{o}C prior to mRNA extraction.

2.4 Microscopy

2.4.1 Lacto phenol trypan blue staining

A 1.5 cm leaf segment from the infected barley leaf was placed into a 2 mL Eppendorf tube, and 1.5 mL of lactophenol trypan blue solution was added to cover the leaf segment. Samples were placed into a beaker of boiling water at 90 ^{o}C for 5 min. The staining solution was removed and replaced with 1.5 mL of chloral hydrate solution to cover the leaf segment. The samples were left to de-stain overnight at room temperature. The leaf segment was removed from the de-staining solution using forceps and placed onto a microscope slide. 200 µL of chloral hydrate was pipetted onto the leaf and covered with a glass cover slip. Samples were viewed using a light microscope.

2.4.2 Sample preparation for confocal microscopy

Leaf segments inoculated with isolate 214-GFP were mounted onto a glass slide using double sided tape to secure the sample. 10-20 µL of silicone oil was pipetted onto the barley leaf surface and a glass cover slip was placed on top. Mounting fluid or cover slip was not required for any dicotyledonous leaf samples.

2.4.3 Confocal laser scanning microscopy (CLSM)

The Leica SP2 confocal microscope, controlled via software Leica Confocal Software (LCS) was used to capture images of 214-GFP strain growth on barley and *N. benthamiana* at an excitation of 488 nm and emission collection of 500-530 nm. At the same time the autofluorescence signal from plant chlorophyll was collected with an emission range of 650-700 nm. The DM6000 microscope was fitted with a FI/RH filter block (excitation filter BP 490/15, dichroic mirror 500, emission filter BP 525/20; excitation filter BP 560/25, dichroic mirror 580, emission filter BP 605/30) and water

dipping lenses (HCX APO L10x/0.30 W U-V-1, L20x0.50 W U-V-1, L40x/0.80 WQ U-V-1 or L63x/0.90 W U-V-1)

2.5 Gene expression

2.5.1 Total RNA, mRNA extraction and cDNA synthesis

Total RNA was extracted from barley leaves, conidia prepared as described above and conidia germinated in sterile distilled water for 24 h using a Qiagen RNeasy Plant mini kit, following the manufacturer's protocol. RNA integrity was tested by agarose gel electrophoresis. The extraction of mRNA from inoculated leaf samples was carried out in accordance with Dynabeads® mRNA DIRECT™ Kit protocol (Invitrogen). RNA concentration was determined using a NanoDrop 1000 Micro Photometer (Thermo Scientific) at wavelength λ260 nm. Prior to cDNA synthesis, RNA samples were DNaseI treated using Ambion Turbo DNA-free™ DNA Removal Kit following the manufacturer's protocol and RNA concentration was measured again as previously mentioned. First strand cDNA for real time RT-PCR was synthesised from 10-15 μg of total RNA or 150 ng of mRNA by oligo dT priming using the SuperScript® III Reverse Transcriptase (Invitrogen), following the manufacturer's protocol.

2.5.2 Quantitative RT-PCR (qRT-PCR)

Primer pairs were designed for each selected candidate effector sequence using the Primer Express software (Applied Biosystems, USA) following the manufacturer's guidelines. Primer pairs were initially tested for specificity using *R. commune* cDNA, gDNA and barley cDNA as template using the Bio-Rad Chromo4 RT-PCR detector and the following thermal cycling conditions: heated to 94 ^{o}C for 1 min, followed by 40 cycles of: 94 ^{o}C for 30 secs, 60 ^{o}C for 1 min and 72 ^{o}C for 30 secs and held at 4 ^{o}C.

Amplification of product of the correct size was confirmed by gel electrophoresis. Any primers that amplified products from barley cDNA were not used for real time RT-PCR assays and new primers were redesigned at a later stage if required. For qRT-PCR assay **e**ach reaction consisted of 6ul of SYBR green mix (Applied Biosystems, USA), 1μL forward primer, 1μL of reverse primer, 1μL of template DNA and 3 μL of H_2O. Amplification efficiency of selected primers was optimised with cDNA from *R. commune*. Optimal concentrations of each primer were determined using the Bio-Rad Opticon monitor software. Concentrations of primers for qRT-PCR were selected based on the highest efficiency at the lowest threshold cycle (Ct). All real time assays included a negative control and were carried out in triplicate. Melting curves were analysed to ensure amplified product was of correct size. *R. commune* actin was used as the endogenous controls.

2.6 Cloning strategy and expression plasmids construction

2.6.1 Preparation of *S. cerevisiae* competent cells

10 mL of yeast extract peptone dextrose media was inoculated with *S. cerevisiae* strain FY824 and cultured at 30 °C in a shaker overnight. 5 mL of overnight culture was used to inoculate 2 flasks each containing 300 mL of YPD broth and allowed to grow at 30 °C to a density (OD_{600}) of 0·6–0.8 (approximately 2 x 10^7 cells/mL). Cultures were aliquoted into 50 mL falcon tubes and cells harvested by centrifugation at 3000 g for 5 min at room temperature. Single Stranded (SS) DNA from salmon sperm (10mg/mL, Sigma-Aldrich) was boiled for 5 min then cooled on ice. Harvested cells were washed once with 50 mL of SDW and centrifuged at 3000 g for 5 min at room temperature. The supernatant was removed and the pellet was washed once with 40 mL of SORB solution and centrifuged at 3000 g for 5 min at room temperature. The supernatant was

removed and cells were re-suspended in a total volume of 360 µL SORB solution and 40 µL of carrier SS DNA (4 °C) per 50 mL starting culture. Cells were aliquoted into 1.5 mL Eppendorf tubes at room temperature and stored at -80 °C.

2.6.2 Preparation of *P. pastoris* electro competent cells

A starter culture of 5 mL of *P. pastoris* in YPD in a 50 mL Falcon tube was grown at 30°C overnight in a shaking incubator. 0.1–0.5 mL of the starter culture was used to inoculate 500 mL of fresh YPD in a 2L flask. The culture was grown overnight again to an OD_{600} = 1.3–1.5. The cells were centrifuged at 1,500 × g for 5 min and re-suspend in 500 mL of SDW. Cells were centrifuged at 1,500 × g for 5 min and re-suspend in 250 mL of SDW. Cells were centrifuged for a further two times and the pellet was re-suspend in 20 mL of 1 M sorbitol and 1 mL of 1 M sorbitol respectively. Cells were used immediately.

2.6.3 Yeast re-combinational cloning (YRC)

YRC was conducted using the procedure described by Oldenburg (Oldenburg et al., 1997). Chimeric primers containing 30 base pair extensions at 5' end of sequence were designed for each fragment to be inserted into the plasmid. PCR was used to amplify each fragment using the Phusion® High-Fidelity DNA Polymerase using the thermocycler conditions provided in table 2.8.1. The amplification of each fragment was confirmed via gel electrophoresis. The PCR products were transformed into *S. cerevisiae* along with a linearised acceptor vector for assembly in yeast via its endogenous recombination system. The correctly assembled plasmids were isolated from yeast. Yeast plasmid DNA was transformed into *E. coli* prior to the cassette or

expression plasmid being recovered. Sequencing was used to confirm correct insertion of all fragments.

2.6.4 Plasmids used for YRC

2.6.4.1 Cloning plasmid

The pRS426 plasmid (ATCC® 77107™) containing the 2-micron origin replication site was used for YRC. The plasmid contains the URA3 gene which is used for the selection of transformants based on restoration of uracil prototrophy.

2.6.4.2 Expression plasmids

The pGAPZα_V5 or pGAPZα_mCh laboratory stocks were used to express proteins of interest in *P. pastoris*. The pGAPZα plasmid from Invitrogen had been modified to include a glycine-alanine linker followed by either V5 or the mCherry epitope. In addition, the URA3 gene and 2-micron origin were also introduced to allow for YRC. To increase the chances of successful secretion in *P. pastoris* the alpha-factor signal peptide replaced the native signal peptide of the genes of interest. To ensure the gene was expressed as a fusion protein the stop codon was removed.

2.7 Transformation protocols

2.7.1 *E. coli* transformation

MAX Efficiency® DH5α™ Competent *E. coli* cells (Invitrogen) were used for all *E. coli* transformations. 50 µL of cells were mixed with 5-10 µg of yeast plasmid, mixed gently and incubated on ice for 30 min. The cell suspension was placed into a water bath at 37 °C for 15 min. The cells were incubated at 4°C for 2 min before 1mL of

super optimal broth (SOC) was added and incubated on a rotating stand for 1-3 hours at 37 °C. Cells were centrifuged at 8000xg for 15 s and 900 µl of supernatant was removed. The remaining cells were plated onto LB plus ampicillin at final concentration of 100 µg/mL for YRC plasmids or Zeocin™ for selection of *E. coli* transformed with pGAPZα expression plasmid (25 µg/mL in low-salt LB). LB selective plates were incubated overnight at 37 °C. Single colonies were genotyped using colony PCR and positive clones were selected to grow in LB overnight.

2.7.2 *S. cerevisiae* transformation

50 µl of competent yeast cells were added to 10µL of DNA mixture (1µL of linearised plasmid pR5426 (100 ng/µl stock) and equal amounts of DNA fragments up to 10µL). 360 µL of PEG/LiAC solution was added to the suspension and vortexed briefly at room temperature. 47 µL of Dimethyl sulfoxide (DMSO) was added to each tube, vortexed briefly and placed into a water bath at 42 °C for 15 min. 1mL of SDW was added and the suspension was centrifuged at 12,000 g for 15 seconds. 1.3 mL of supernatant was taken out and the cells were re-suspended in the remaining 150µL of sample. Twenty percent of the total volume was plated onto SC-ura agar medium and the remaining solution was added to 20 mL of liquid SC-ura medium and incubated on a shaker at 180 rpm, 30°C for 2-3 days. Selection in *S. cerevisiae* is based on restoration of uracil prototrophy.

2.7.*3 P. pastoris* transformation

Following the protocol described in the Invitrogen Pichia expression kit manual, 80 µL of electrocompetent cells were mixed with 5–20 µg of linearized DNA (in 5–10 µL TE Buffer) and transferred into an ice-cold 0.2 cm electroporation cuvette. The cuvette was incubated on ice for 5 min and the cells were electroporated according to the parameters for yeast (*S. cerevisiae*). 1 mL of ice-cold 1 M sorbitol was added immediately to the cuvette and the contents were transferred to a sterile micro centrifuge tube. 200–600 µL of aliquots were plated onto low salt YPD agar medium with Zeocin™ (50 µg/mL). Plates were incubated at 30 °C for 2- 3 days until colonies appeared.

2.7.4 Electroporation transformation of *R. commune*

R. commune conidia were harvested using the previously described method. The pellet obtained from centrifugation was suspended in 10 mL of SDW with 10 µl of ampicillin and left in the dark for 24-48 hours at 17°C for the conidia to germinate. The conidial suspension was washed 3 times with 10 mL of 1 M sorbitol and centrifuged at 1600xg for 3 min. The pellet was re-suspended in 100 µL of 1M sorbitol, transferred to an ice cold 2 mL Eppendorf tube containing 1 µg of DNA and mixed gently. The mixture was kept on ice for 5 min before being transferred to an ice cold electroporation cuvette. The germinated conidia and DNA were electroporated at 1.25 kV and transferred into a 50 mL falcon tube with 10 mL of Potato Dextrose Broth (PDB), 1mL of sorbitol, 10 µL of 100 mg/mL ampicillin and placed onto a rolling shaker for 24 hours. The suspension was centrifuged at 700 g for 5 min and re-suspended in 2 mL of PDB and 1mL of 1M sorbitol. The sample was plated onto CZV8CM agar medium, containing 100 µg/mL of hygromycin and ampicillin. After 2-3 weeks, antibiotic resistant colonies were transferred onto fresh medium containing antibiotics as stated above.

2.7.5 Transformation of fungal protoplasts

2.7.5.1 Protoplast preparation

R. commune conidia were used to inoculate 15- 20, 200 mL bottles of PDB. The spores were left to germinate in the dark at 19 °C for a period of 5-6 days until growth of mycelium was visible. The mycelium was collected using a 60 µm mesh and washed carefully with 20 mL of KC solution. Using sterile spatula, the mycelium was transferred into a 50 mL centrifuge tube containing 30 mL of filter sterilised protoplasting solution (5 mg/mL lysing enzymes in KC). The falcon tube was incubated at room temperature with gentle shaking in the dark for 3-3.5 hours. To decrease the loss of viability the remainder of the procedure was carried out quickly. The digested mycelium was filtered through a 100 µm nylon mesh, and the filter was washed with 5 mL KC. Gentle agitation with a sterile pipette tip prevented the filter from becoming clogged. Filtered protoplasts were passed through a 30 µm mesh and the filter was washed with 10 mL KC. Protoplasts were centrifuged in a swing bucket rotor at 700 g for 4 min. Supernatant was removed using a pipette and gently re-suspended in 10 mL KC. Protoplasts were centrifuged and re-suspended a further three times using the same parameters but using 10 ml of KC-MT, 10 mL MT and 1 mL of MT to re-suspend the pellet in the second, third and fourth wash, respectively. Protoplasts were re-suspended using a 5 mL and a pipette tip was used to gently wash/scrape side of tube to release stuck protoplasts. A concentration of 1-5 x 10^7 /mL protoplasts was used for transformation.

2.7.6.2 Protoplast Transformation

The protoplast suspension was mixed with 1 µg of each DNA fragment in a 1.5 mL Eppendorf tube. 500 µl of PEG 6000 in B2 buffer was added and mixed carefully.

Protoplasts were transferred into a 25 mL polystyrene universal tube and DNA mix was added. The tube was mixed gently by rolling the tube for 30-60 seconds and allowed to stand for 5 min. 1 mL of freshly prepared 50% PEG 3350 was added slowly, drop by drop whilst rolling tube at an angle. The tube was rotated for a further 30 seconds, allowed to stand for 2 min, mixed by inverting the tube once and then allowed to stand for 5 min. 2 mL of PDB-(Sucrose)-Mannitol (PDB-(S) M) was added gently and mixed once by inversion and allowed to stand for 2 min. 6 mL of PDB-(S) M was added and mixed once by inversion before standing for 3 min and then mixed again by inversion. The protoplasts were transferred into a 90 mm diameter Petri dish containing 12 mL PDB-(S) M and 12 µL of ampicillin to a final concentration of 50 µg/ml. The dish was sealed with Nescofilm and left to incubate at room temp for 48-72 hours. Regenerated protoplasts were transferred into a 50 mL falcon tube. 5-10 mL of PDB-(S) M was used to remove any protoplasts which had adhered to the Petri dish and transferred them to the tube. The regenerated protoplasts were centrifuged at 700 g for 5 min and the supernatant was removed using a 5 mL pipette. The pellet was re-suspended in 2 mL of PDB-(S) M and spread onto 10-15 x 90 mm diameter Petri dishes of CZV8CM containing 100 µg/mL of hygromycin and incubated at 19-20 ºC in the dark.

2.7.6 Screening of yeast transformants by colony PCR

Selected colonies were picked using a sterile pipette tip and transferred to a new selection plate, with the remainder of the colony re-suspended in a 0.5 mL PCR tube with 50 µL of 0.02 M Sodium Hydroxide (NaOH) and vortexed briefly. Samples were incubated at 95 °C for 30 min. 1µL of the supernatant was used as the PCR template in a 20 µL reaction.

2.7.7 Protein expression analysis in *P. pastoris* cultures

Strategies for analysing expression in selected clones are described in detail in the Pichia expression kit manual. Approximately, 8 clones were chosen to allow for a representative range of expression levels. Positive clones were cultured in YPD for 48 – 72 hours at 30 °C on a shaker at 200 rpm. The cells were pelleted at 2000 g for 5 min and the supernatant was analysed for protein expression using high-sensitivity SYPRO ® Ruby Protein Gel Stain (Invitrogen) or immunoblotting. Once the expression was confirmed the transformed cells were grown in large volumes of PDB to harvest the culture supernatant.

2.7.7.1 Protein concentration

Culture supernatant containing v5 tagged protein was concentrated using Vivaspin 20 (MWCO 10 000) columns under the manufacturers guidelines. Buffer exchange was carried out using 3 washes of HN buffer and a final wash with HNT buffer

2.7.7.2 Protein purification

V5 tagged proteins were concentrated using Anti-v5-tga mAb – magnetic beads from MBL following the manufacturers guidelines, except at the last stages where the beads

were re suspended in HNT buffer (not boiled), placed against the magnet and the supernatant removed and stored at -20.

2.8 Molecular biology protocols

2.8.1. Fungal & plant DNA extraction

Fungal mycelium from *R. commune* isolates was obtained by scraping the surface of 14 day old plates. A mortar and pestle with the addition of liquid nitrogen was used to disrupt the fungal mycelium. DNA extraction was carried using a QIAGEN DNeasy Plant Mini kit following the manufactures guidelines

2.8.2 Plasmid extraction

2.8.2.1 Plasmid extraction from *E. coli*

After growing transformed *E. coli* in liquid LB medium overnight, cells were centrifuged at 8000 g for 3 min at room temperature. *E. coli* transformed cells were extracted following the protocol from the QIAprep® Miniprep Handbook. PCR was used to amplify the constructed cassette and PCR products were purified using either the MinElute PCR Purification Kit (QIAGEN) following the manufacture's protocol or the Wizard® SV Gel and PCR Clean-Up System (Promega) also using the manufacture's guidelines. Sequencing was used to verify the constructs prior to further use.

2.8.2.2 Plasmid extraction from yeast cells

Transformed cells were harvested from the media and plasmids were rescued from *S. cerevisiae* based on a method by Robzyk *et al*., 1992. Briefly, the pellet was re-suspended in 100µL of Sodium chloride-TRIS-EDTA-Triton (STET) buffer and ~0.3 g of 0.45 mm acid washed glass beads (Sigma) were added before vortexing vigorously

for 5 min. Another 100 µL of STET was added, vortexed briefly and placed into a water bath at 95 ^{o}C for 2 min. The mixture was cooled briefly on ice and centrifuged 13,000 g for 10 min at 4°C. 100 µl of the supernatant was transferred to a fresh tube containing 50 µL of 7.5 M ammonium acetate and incubated at -20 °C for 1 hour and spun at 13,000 g for 10 min at 4°C. 100 µl of the supernatant was added to 200 µL of ice-cold ethanol with 10 µL 3M sodium acetate, and centrifuged at 16, 000 g for 20 min at 4 °C. After removing the supernatant 200 µl of 70% ethanol (EtOH) was added, centrifuged at 16,000 g for 5 min at 4 °C. Supernatant was removed, pellet was allowed to dry then re-suspended in 20 µL of SDW. On other occasions Zymoprep™ Yeast Plasmid Miniprep extraction kit (Zymo Research, USA) was used following the manufacturers protocol.

2.8.2 Polymerase chain reaction

Polymerase chain reactions (PCR) were carried out using the Biorad T100^{TM} Thermal cycler. The PCR cycle was dependent on the Tm of the primers, template, amplicon size and type of polymerase used. The different polymerases and cycles are shown in table 2.1.

Table 2.1 PCR conditions for Phire Polymerase(a), Phusion Polymerase(b) and GoTaq(c) Polymerases

Cycle step	3 Step Protocol		Cycles
	Temperature	Time	
Initial denaturation	98°C	30secs - 2mins	1
Denaturation Annealing Extension	98°C 45-72°C 72°C 72°C	5s 5s 10-30s/kb[a], 10-15s/kb[b] or 1 min/kb[c]	25-35
Final Extension	72°C 4°C	1 min hold	1

2.9 Proteomics protocols

2.9.1 Protein extraction

Leaves were placed into a mortar, covered with liquid nitrogen and ground to a fine powder. Extraction buffer in a 1:1 ratio of wt/vol was added and plant leaf material was further ground ensuring no thawing occurred. Samples were centrifuged at 8000 g for 5 min at 4 °C and the supernatant was transferred to 1.5-mL microcentrifuge tube and used immediately for enzymatic and protein assays.

2.9.2 Protein visualisation

2.9.2.1 SDS-polyacrylamide-gel electrophoresis

1-4 µL of protein sample was combined with 5 µL Novex® Tricine SDS Sample Buffer (2X), 1 µL of NuPAGE® Reducing Agent (10X) or 20 mM DTT and up to 4 µL of deionised water to a total volume of 10 µL. Samples were heated at 95° C for 15 min and separated with gel electrophoresis using Novex® Pre-Cast Gel chamber (Invitrogen). SYPRO ® Ruby Protein Gel Stain (Invitrogen) was used to visualise proteins and sizes were determined by comparing the migration of the protein band to a molecular mass standard.

2.9.1.2 Western blotting

Proteins were transferred from the SDS gel onto a nitrocellulose membrane (Millipore) for 90 min at 200 mA using an XCell SureLock® Mini-Cell and XCell II™ Blot Module. After washing twice with water, the membrane was incubated with gentle agitation in blocking buffer for 1 h at room temperature to reduce unspecific binding. The membrane was then incubated with Anti-V5 Antibody conjugated with horseradish peroxidase (HRP) from ThermoFisher Scientific, which was diluted in blocking buffer (1:10000) for 1 h at room temperature. The membrane was washed twice for 5 min with Phosphate-buffered saline (PBS) plus Tween (0.5%) and with a final wash of PBS. The protein bands were detected using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific) and visualized on Xograph compact X4 developer.

2.9.3 Proteome analysis of apoplast from barley cultivars Optic, Atlas and Atlas46

2.9.3.1 Apoplastic fluid extraction of barley leaves

Apoplastic fluid was extracted using vacuum infiltration as described with slight modifications (Vanacker, H *et al*., 1998; Bolton *et al*., 2008). 8-10 day old cotyledons were gently removed from the plant stem. Approximately 20 leaves were placed into a 2 litre glass beaker and covered with SDW. A second smaller glass beaker was placed on top of the leaves to prevent them rising. Vacuum was applied until the leaves were completely infiltrated using a vacuum infiltrator / freeze drier (Edwards Modulyo). The infiltrated leaves were blotted dry with paper tissue and were rolled in muslin cloth and placed leaf tip first into a 20 mL syringe which was introduced into a 50 mL conical tube. The apoplast extract was collected by centrifuging at 1000 g for 15 min at 4 °C. The fraction collected in the 1.5 mL tube was transferred to a new 2 mL Eppendorf tube and centrifuged again for 10 min at 1600 g at 4 °C. The supernatant was decanted into a clean 1.5 mL tube and filter sterilised using 0.2-µm Whatman filter. The samples were concentrated to approximately 1/5th of their original volume and stored at -80 °C.

2.9.3.2 Enzyme contamination assay

To test the level of cellular contamination of apoplastic fluid, the activity of glucose-6-phosphate dehydrogenase (G6PDH) was analysed. For each 1mL sample reaction 0.89 ml of potassium phosphate buffer, pH7.0 (0.1 M), 50 µL of 6 mM NADP and 10 µL of plant or apoplast extract were combined. The reaction was started by addition of 50 µL glucose-6-phosphate (40nM). Homogenised leaf material was used as a positive control. In addition, the extracted apoplastic fluid was spiked with known quantities of enzyme: 1µL of 5 mg/mL solution of G6PDH 550-1100 ng protein/mol. The enzyme activity was measured as the rate of increase in absorbance at 340 nm (ΔA340) per min. One unit was defined as the amount of enzyme that produces 1 µmol of NADPH per min for G6PDH.

2.9.3.3 Sample preparation for mass spectrometry (MS) analysis: In gel digestion used for *R. commune* apoplastic studies

10-20 ng of protein was loaded onto SDS-polyacrylamide-gel and separated following the previously described procedure. Each lane of loaded protein samples was divided into three and each of the pieces were further divided into 9 equally sized pieces. This approach allowed for the enhancement of lower abundant proteins by reducing the number of overall proteins within the sample. The gel pieces were washed with 100 µl 100 mM NH_4HCO_3: 100 % acetonitrile (ACN) for 10 min, at room temperature on a shaker. The solution was removed and this step was repeated. 50 µL of 100 % ACN was added and the gel pieces changed to white in colour before the addition of 50 µL of 100 mM NH_4HCO_3 and incubated at 37 °C for 30 min on a shaker. The solution was removed and the gel pieces were dried completely in a vacuum centrifuge at a maximum temperature of 45 °C for 1-3 hours. 50 µL of 10 mM DTT solution was added to the dried gel pieces and incubated at 55 °C for 45 min in a heated shaker. The DTT solution was removed and 50 µL of 55 mM of iodoacetamide solution was added, incubated at room temperature in the dark for 30min. Iodoacetamide solution was removed and gel pieces were washed with 100 mM NH_4HCO_3/100% ACN for 10 min at room temperature on a shaker. This step was repeated twice. The gel pieces were dried completely as mentioned above and 10-20 µL of diluted trypsin solution was added (10 µl of 1 µg/µL trypsin in 490 µl of 50 mM NH_4HCO_3, pH8) and the tubes were sealed to prevent evaporation. After an overnight incubation at 37 °C, 20 µL of 0.1 % Trifluoroacetic acid (TFA) and 20 µL of 100 % ACN were added and the gel pieces were sonicated in a bath of ice water for 15 min. The supernatant was removed and pipetted into a new Eppendorf tube. 100 µL of 30 % ACN: 0.1% TFA was added to the remaining gel pieces and they were sonicated as before. The supernatant was

removed and added to previous supernatant. 100 µL of 50 % ACN: 0.1 % TFA was added to the gel pieces and sonicated again before the supernatant was removed and added to the pooled supernatant. The total volume of supernatant was reduced to approximately 100 µL in a vacuum centrifuge at 60 °C. Samples were cleaned using C18 Ziptip columns (Millipore).

2.9.3.4 Sample preparation for MS analysis: In solution digestion used for quantitative plant apoplastic fluid proteome analysis

5µl of 45 mM DTT (in 25 mM NH_4HCO_3, to a final working concentration of 10 mM) was added to 100 µg of protein sample and incubated at 50 °C on a heated shaker for 15 min. The mixture was cooled slightly before the addition of 5 µL of 100 mM IAA (diluted in 25 mM NH_4HCO_3) and incubated in the dark at room temp for 15 min. 1:100 enzyme to substrate m/m ratio of trypsin was added and incubated overnight at 37 °C. Samples were cleaned using C18 Ziptip columns (Millipore).

2.9.3.6 Sample cleaning using C18 Ziptip columns

Using 500 µl of POROS reversed-phase packing and 700 µl of 70 % ACN: 0.1 % TFA a slurry was prepared and stored at 4 °C. A blunt ended needle was used to push a small circle of C18 filter disk into a gel loading tip to act as a stopper. 10µL of 0.1% TFA was loaded into the tip, then 7 µL of the slurry and pushed through the tip until the slurry was packed into a column. Ziptips were stored at 4 °C.

The column was activated by adding 20 µL 50 % ACN: 0.1 % TFA being careful not to remove all the liquid to prevent the column from drying out. The excess on the column was washed with 20 µL of 0.1 % TFA. The maximum of 60 µL sample was loaded onto the column. A 1 mL syringe was used to add pressure to get the sample through the column and the flow through was put back into the sample Eppendorf tube. Unbound waste was washed away with 20 µL of 0.1 % TFA and the bound peptides were eluted from the column using 40 µL of 50 % ACN: 0.1 % TFA into a new 0.5 mL Eppendorf tube twice, to give a total final volume of 80 µL. The samples were dried to approximately 10 µL using a vacuum centrifuge at 60 °C.

2.9.3.7 Dimethyl labelling procedure

To analyse the apoplastic protein samples quantitatively, dimethyl labelling was used following the on column procedure published by Borsema *et al*. (2009). 10 µg of protein sample was digested in solution as previously described and dried by vacuum centrifugation at 1500 rpm at room temperature for 30 min. The remainder of the procedure was completed in a fume hood due to the toxic vapours of the labelling reagents. Each of the following dimethyl labels were prepared according to the protocol with the correct isotope combinations of formaldehyde and cyanoborohydride to generate the light, intermediate and heavy dimethyl labels and kept on ice. The samples were reconstituted in 1 mL of 5 % formic acid. SepPak columns were washed with 2 ml of ACN and twice with 2 mL of Reversed Phase (RP) solvent A. Samples were loaded onto the columns and washed with 2 mL of RP solvent A. Each column was flushed five times with 1 mL of the respective labelling reagent (light, intermediate or heavy). Next, the columns were washed with 2 mL of RP solvent A. The samples were eluted

with 500 ml of RP solvent and collected into an Eppendorf tube. The differentially labelled samples were mixed and analysed using MS.

2.10 Polysaccharide binding assay

A polysaccharide affinity precipitation assay was used to determine the affinity of LysM domain containing proteins to various polysaccharides: crab shell chitin, chitosan, xylan or cellulose (all from Sigma Aldrich). 5 mg of polysaccharide was added to 800 µl of SDW. The polysaccharide solutions were mixed with 10 µg/mL of each protein. AVR4 and ECP6 provided by Wageninen University, The Netherlands were used as controls. After an overnight incubation at 4 °C on a rolling shaker, the insoluble fraction was pelleted by centrifugation (5 min, 13,000 × g) and the supernatant was collected. Using SDW the insoluble fraction was washed three times. Both supernatant and pellet were examined for the presence of protein using SDS-polyacrylamide gel electrophoresis.

2.11 *In vitro* fungal growth assays

Fungal cell wall protection against chitinase assay was performed as described previously (van den Burg et al., 2006; van Esse et al., 2007; de Jonge et al., 2010). 40 µL of *T. viride* spores in PDB at a concentration of $1x10^3$ were incubated overnight at room temperature on a 96 well microtiter plate. Candidate effector proteins, produced in *P. pastoris* were added to the conidial suspension at a final concentration of 30 µM and incubated at room temperature for 2 hours. 5 µL of crude tomato extract was added. After 4 hours of incubation, the growth of *T. viride* was analysed microscopically.

2.12 Bioinformatic analyses

2.12.1 Sequence analysis tools

BLAST online tool was used for amino acid and nucleotide sequence comparisons to the sequence databases. Statistical significance of matches are also calculated.

https://blast.ncbi.nlm.nih.gov/Blast.cgi

SignalP 4.1 server was used for the prediction of the presence and location of signal peptide cleavage sites in amino acid sequences from eukaryotes, based on a combination of several artificial neural networks. http://www.cbs.dtu.dk/services/SignalP/

TargetP 1.1 was used for the prediction of subcellular location of eukaryotic proteins. http://www.cbs.dtu.dk/services/TargetP/

TMHMM Server v. 2.0 was used for the prediction of transmembrane helices in proteins. http://www.cbs.dtu.dk/services/TMHMM/

Praline multiple alignment tool was used to determine similar DNA and amino acid sequences. http://www.ibi.vu.nl/programs/pralinewww/

Phylogeny.fr was used to construct robust phylogenetic tree from sets of sequences. (Dereeper et al., 2010) http://www.phylogeny.fr/

2.12.2 Proteomic analysis

MaxQuant software was downloaded and used for the analysis of mass-spectometric data sets http://www.biochem.mpg.de/5111795/maxquant downloaded

Perseus software was downloaded and used as a statistical tool to interpret protein quantification, interaction and post-translational modification data. http://www.coxdocs.org/doku.php?id=perseus:

2.12.3 Genome databases

PlantsDB Hordeum vulgare database was used to obtain sequence information from the http://pgsb.helmholtz-muenchen.de/plant/barley/:

(Hordeum vulgare_IBSC2012_V3_GENOMIC.fa with a standard e- value of 10e-5)

The Rhynchosporium database from HelmholtzZentrum muchen - The German Research Centre for Environmental Health was used to obtain all *R. commune* sequence data

http://pedant.helmholtzmuenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_t914237_Rhy_commu_UK7_v2

CHAPTER 3

3. Identification and Characterisation of *R. commune* Candidate Effectors

3.1 Introduction

The first step of the journey to gain an understanding of how *R. commune* colonises its host and evades barley immunity begins with the elucidation and characterisation of the pathogens' effector repertoire. Rapid evolution of effector molecules has allowed for the transition and continuation of *R. communes'* status as a pathogen, in turn leading to devastating crop losses (Chisholm *et al*., 2006). However, apart from the three necrosis inducing peptides (NIPs) mentioned previously, the composition and function of the *R. commune* effector repertoire remains unexplored despite the agronomic importance of this pathogen.

Increasing affordability of genome sequencing has revolutionised the quest to identify pathogen genes that function in aiding the infection process. There are now over fifty fungal phytopathogen genomes available online (CPRG, 2016), but there are numerous on-going sequencing projects, that are not publicly available yet. Although the task of identifying genes of interest from genome sequence data alone can be daunting, the development of bioinformatics approaches for effector identification has resulted in an enhanced and more expeditious way of identifying candidates (Win *et al*., 2006; Torto-Alalibo *et al*., 2009; Perseden *et al*., 2012; Van Weymers *et al*., 2016).

A fundamental part of effector discovery is characterisation of gene expression during infection. Analysis of transcript abundance is a useful method to prioritise candidates (Cooke *et al*., 2012). In this study, quantitative reverse transcription polymerase chain reaction (qRT- PCR) provided an effective method to obtain levels of transcript abundance of the selected candidates throughout the infection process. The same strategy was used to reveal the expression of the three necrosis inducing peptides (Kirsten *et al*, 2012). To date there have been numerous research articles extrapolating the expression of potential effectors (Oh *et al.,* 2009; Fabro *et al*., 2011; Bhadauria *et al*., 2015; Petre *et al*., 2015). Studies have mainly focused on the expression of genes *in planta* during infection however, it is not uncommon for some pathogen effectors to be secreted during vegetative growth and in some instances, effectors have been first discovered through their expression in synthetic media. In fact, all of the NIPs were identified initially in *R. commune* culture filtrate (Wevelsiep *et al*., 1991).

However, validation of effector expression can only be fully achieved through the identification of the protein during infection. This information provides experimental continuity between genome sequence information and expression data. Using a proteomics approach to further identify and characterize candidate effectors can be a useful tool (Fernandez Acero *et al*., 2009; Brown *et al*., 2012; Delaunois *et al*., 2014; Lu & Edwards, 2016). Nevertheless, the pinnacle of effector discovery is achieved by defining the function of candidate effectors. In filamentous fungi, a common application to analyse the function of a gene is by replacement or disruption with a marker gene for antibiotic resistance (Yang *et al*., 2004; Kück & Hoff, 2010; Chung & Lee, 2014). Targeted gene disruption or replacement can be achieved through manipulation of the DNA repair mechanism- homologous recombination (Ruiz-Diez *et*

al., 2002). *R. commune* has been successfully transformed previously using *Agrobacterium tumefaciens*-mediated transformation (ATMT) to express a cytoplasmic fluorescent marker (Thirugnanasambandam *et al*., 2012). In addition, *R. commune* NIPs deletion mutants were generated and analysed for their ability to infect different barley cultivars (Kirsten *et al*., 2012). Targeted gene disruption for NIP1 and NIP3 used protoplast transformation with the efficiency of this method ranging from 0.85 to 3.3 %. Using ATMT transformation technique for the deletion of NIP2 resulted in a rate of 1 in 50 successful gene knock outs. The gene knockouts led to varying quantitative effect on pathogenicity depending on the host genotype (Kirsten *et al*., 2012).

Taking into consideration the research that has previously been conducted, the aim of the study was to analyse candidate effector sequences and characterise expression of the selected candidate effectors during infection to help prioritise them for further functional characterisation.

3.2 Results

3.2.1 Background on selected candidate effectors and confirmation of sequence analysis from predictive pipeline

Prior to the commencement of the project, the *R. commune* genome (strain 13-13) was sequenced and to identify candidate efectors a predictive pipeline was used. This led to identification of 61 candidate effectors which were composed of 59-225 amino acid residues, containing at least 4 cysteines with no transmembrane domain and were potentially specific to this pathogen as originally, they did not match any sequences in NCBI (http://www.ncbi.nlm.nih.gov/) database (Table 3.1). In addition, predicted *R. commune* effector gene sequences were screened for the presence/absence and/or single

nucleotide polymorphisms (SNPs) in genome sequences of 9 *R. commune* strains with different race specificities, including contemporary isolates, originating from several different sites across the UK, as well as a super-virulent strain from Australia (AU2). This allowed prediction of effectors that are present in all strains and are largely conserved in *R. commune* populations (Avrova, unpublished data).

Sequence conservation of predicted *R. commune* effectors is likely to be critical for potential durability of *R* genes recognising these effectors hence, 22 *R. commune* candidate effectors which were the least variable between *R. commune* isolates were selected. As the data was generated via predictive pipelines it was important to confirm the sequence analysis prior to determining the transcription profiling of the selected candidates during infection. Details of all analysis are summarised in Table 3.1.

SignalP (http://www.cbs.dtu.dk/services/SignalP/) determines the presence of a cleavage site indicating the high possibility of a signal peptide. The signal peptide is present at the N-terminus of the majority of newly synthesised proteins destined towards the secretory pathway (Lodish *et al*., 2000). All candidates contained a cleavage site and predictions of a signal peptide were confirmed except for *Rc_05049* (D=0.346, which is below the D-cut-off=0.450 used by Signal-4.1). Further analysis using TargetP (http://www.cbs.dtu.dk/services/TargetP/) localisation predictor confirmed the signal peptide predictions. However, *Rc_05049* which was predicted to contain no signal peptide was still predicted to be part of the secretory pathway. The localisation results revealed candidate *Rc_05673* contained a targeting peptide which locates it to the mitochondria. The reliability score is the size of the difference between the highest and second highest output scores. Thus, the lower the value of RC the safer

the prediction. All candidate predictions based on the reliability score were between RC=1 and RC=2 indicating a strong prediction.

3.2.3 Homology of *R. commune* candidate effectors to other fungal proteins

Originally the candidate effectors used in this research were selected as *R. commune*-specific as they did not match any proteins in NCBI database, but as many more fungal genomes have been sequenced in the last 3 years the NCBI BLASTp search was repeated to identify any homology to other predicted proteins. Sequence similarities were considered significant if the expected E value was less than or equal to 1e-04.

Candidate effector *Rc_6721* matched a putative aldehyde dehydrogenase (80% sequence identity) from the fungal plant pathogen *Diaporthe (Phomopsis*) species disease complex. Aldehyde dehydrogenases (ALDHs) metabolize endogenous and exogenous aldehydes and thereby mitigate oxidative damage in prokaryotic and eukaryotic organisms (Singh *et al*., 2012). The expression of this protein during the early stages of infection may signify a role in detoxification of the plant apoplast (Zang *et al*., 2012). Another eight candidate effectors matched hypothetical proteins from other fungi (Table 3.1). The remaining 13 candidate effectors did not match any sequences in NCBI database.

Most of BLASTP matches were to protein sequences from the foliar fungal endophyte *Phialocephala scopiformis*. In addition, there were similarities between some of the candidates to hypothetical proteins from *Marssonina brunnea* an important fungus that causes Marssonina leaf spot on all species of *Populus*, the soil borne pathogen *F.*

oxysporum and a fungal plant pathogen that causes root rot in flax and wheat *Microdochium bolleyi*.

Table 3.1. Sequence analysis of *R. commune* candidate effectors and homology to other fungal proteins

candidate gene Id	*Protein length*	*Cysteines*	*localisation*	*Top BLASTp hit*	*Species*	*Accession #*	*e value*
Rc_01097	*103*	*8*	*s*	*hypothetical protein MBM_09244*	*Marssonina brunnea f. sp.*	*XP_007297133.1*	*2e-5*
Rc_01130	*157*	*14*	*s*	*No significant similarities*			
Rc_01776	*91*	*8*	*s*	*hypothetical protein FOCG_15424*	*Fusarium oxysporum f. sp.*	*EXL42069.1*	*4e-5*
Rc_02091	*138*	*10*	*s*	*No significant similarities*			
Rc_02410	*149*	*6*	*s*	*No significant similarities*			
Rc_2835	*125*	*6*	*s*	*No significant similarities*			
Rc_05049	*194*	*4*	*s*	*No significant similarities*			
Rc_05109	*116*	*6*	*s*	*hypothetical protein LY89DRAFT_729122*	*Phialocephala scopiformis*	*KUJ21607.1*	*9e-33*
Rc_5673	*157*	*8*	*m*	*No significant similarities*			
Rc_05783	*121*	*6*	*s*	*hypothetical protein LY89DRAFT_579580*	*Phialocephala scopiformis*	*KUJ20421.1*	*1e-48*
Rc_06721	*104*	*8*	*s*	*putative aldehyde dehydrogenase*	*Diaporthe ampelina*	*KKY34992.1*	*2e-17*
Rc_07354	*151*	*8*	*s*	*hypothetical protein LY89DRAFT_723264*	*Phialocephala scopiformis*	*KUJ10661.1*	*2e-18*
Rc_07612	*129*	*8*	*s*	*No significant similarities*			
Rc_08075	*160*	*6*	*s*	*hypothetical protein MBM_08646*	*Marssonina brunnea f. sp.*	*XP_007296535.1*	*8e-47*
Rc_08731	*145*	*8*	*s*	*hypothetical protein Micbo1qcDRAFT_180629*	*Microdochium bolleyi*	*KXJ85649.1*	*1e-5*
Rc_10317	*67*	*6*	*s*	*No significant similarities*			
Rc_10933	*137*	*8*	*s*	*No significant similarities*			
Rc_10934	*117*	*6*	*s*	*No significant similarities*			
Rc_11163	*126*	*4*	*s*	*hypothetical protein LY89DRAFT_730227*	*Phialocephala scopiformis*	*KUJ21448.1*	*1e-35*
Rc_11301	*191*	*7*	*s*	*No significant similarities*			
Rc_11752	*59*	*6*	*s*	*No significant similarities*			
Rc_11935	*93*	*5*	*s*	*No significant similarities*			

3.2.4 *R. commune* candidate effector gene expression in *planta and in vitro*

3.2.4.1 Analysis of candidate effector expression in *R. commune* strain L2A during infection in *planta*

In order to initially select candidate genes which are highly expressed during a compatible interaction for further characterisation an infection time course using strain L2A on susceptible barley cultivar Optic was set up. mRNA extraction was carried out on each of the samples obtained from the infection time course. Expression profiles were obtained using the comparative quantification algorithm - ΔΔCt method and comparing the results from experimental samples with a calibrator (*R. commune* conidia) and normalised against the levels of expression of an endogenous control (Actin).

The candidate effectors expression profiles were split into four groups, dependant on the highest point of expression during the infection (Figure 3.1, 3.2). The largest proportion of candidate effector genes were upregulated at the biotrophic stage. At 1-2 dpi, which represents conidia and germinating conidia, three candidates were identified with a fold increase of over 200 for *R_5109* and *Rc_2410* (Figure 3.2 & Figure 3.3). The next most common peak of expression was at 3dpi during the penetration phase. This group contains five candidates – *Rc1097, Rc11935, Rc_1130, Rc_6721* and *Rc_5049* with the highest expression level at a 137-fold increase for *Rc_6721* (Figure 3.2 & Figure 3.3). Exactly half of the candidate genes were most highly expressed between 6-8 dpi when the fungus would have already established a mycelial network within the apoplast (Figure3.7 D). All of the candidates within this group exhibited a similar profile- a gradual increase from 1-2 dpi with a distinct maximum between 3-6 dpi, continuing expression at 8dpi and a subsequent decline. There was a variance of transcript

abundance ranging from 4 to 1235-fold increase for *Rc_2835* (Figure 3.2 & Figure 3.4). Within the biotrophic groups there were a few cases where a double peak of gene expression occurred for genes *Rc_1130* - group 2, and *Rc_8731* from group 3 (Figure 3.3). The second peak was identified in the necrotrophic stages. Only three candidates, *Rc_8075*, *Rc_10934* and *Rc_11163* were found to increase their transcript abundance from the early stages of necrotrophy (Figure 3.2 & Figure 3.3).

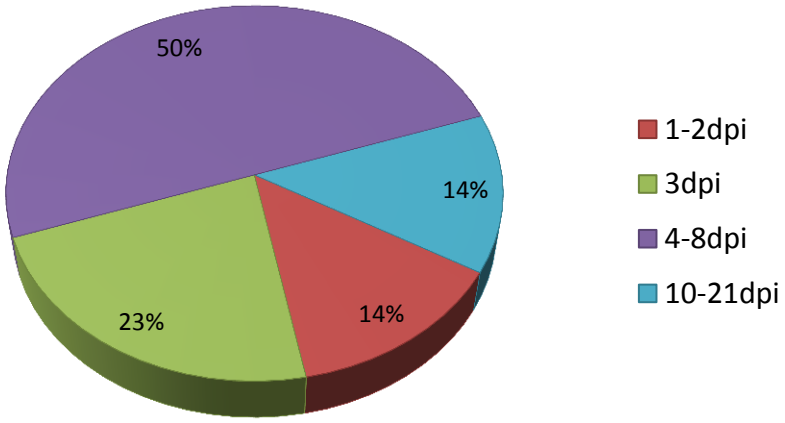


Figure 3.1: Pie chart representing the percentage of highest level of transcript abundance of *R. commune* candidate effectors at different time points of infection in planta; germination 1-2dpi, biotrophic stage 3-8dpi and necrotrophic stage 10-21dpi

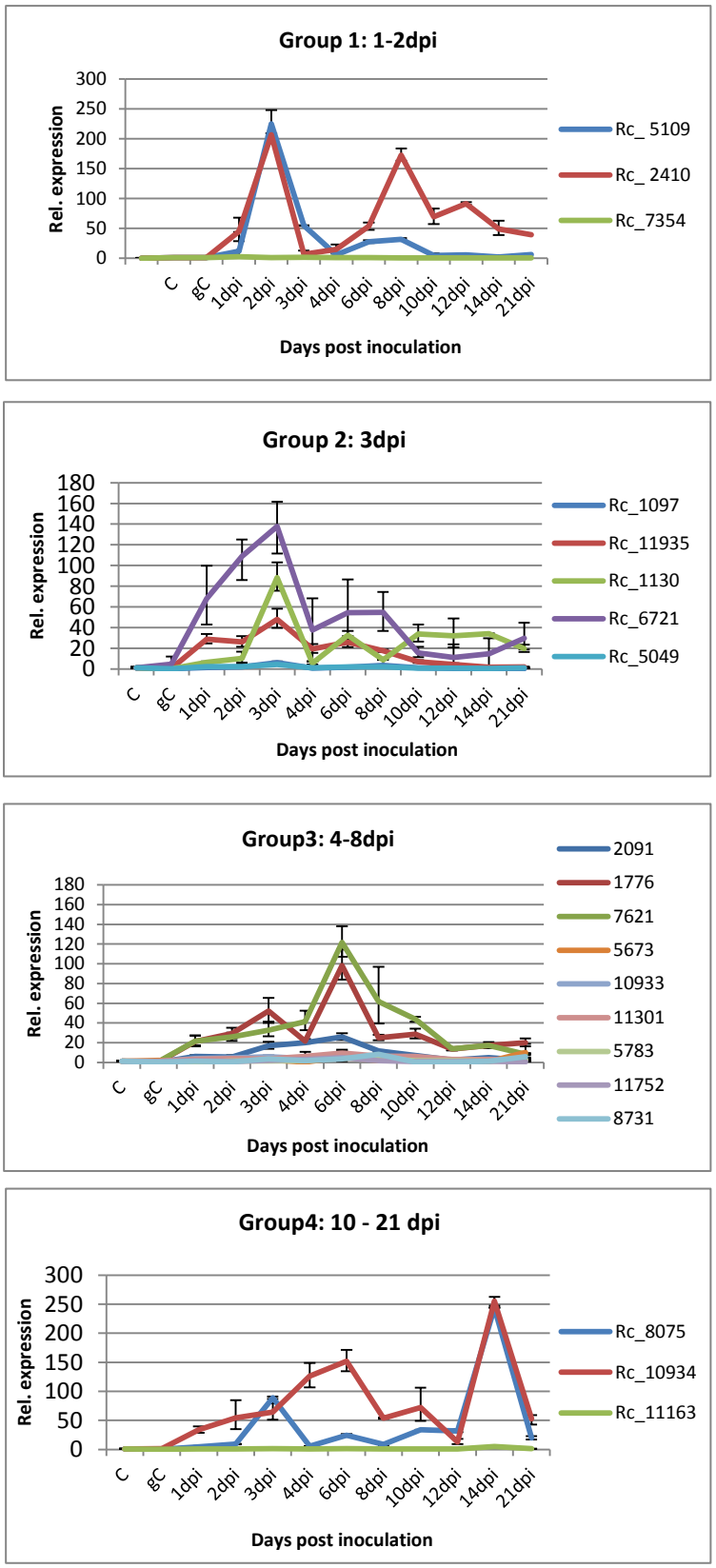


Figure 3.2: Relative transcript abundance of *Rhynchosporium commune* candidate effectors during infection of susceptible barley cultivar Optic with *R. commune* strain L2A normalised against *R. commune* endogenous control Actin. Error bars indicate confidence intervals of the 3 technical repetitions

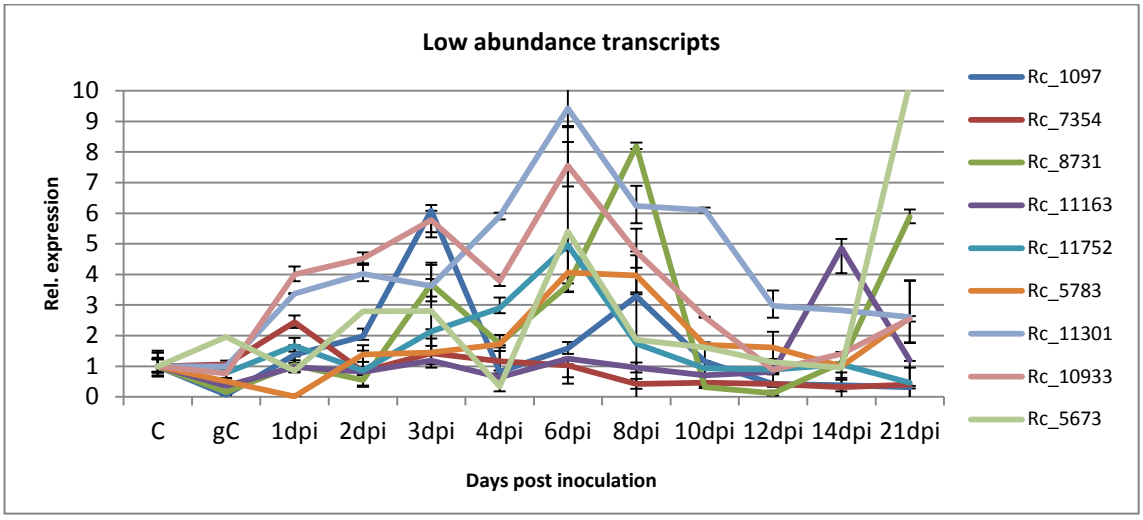


Figure 3.3: Relative expression of low abundance transcript of *Rhynchosporium commune* candidate effectors during infection of susceptible barley cultivar Optic with *R. commune* strain L2A normalised against *R. commune* endogenous control Actin. Error bars indicate confidence intervals of the 3 technical repetitions. *Rc_7354* - Group1, 1-2 dpi; *Rc_1097* – Group 2, 3dpi; *Rc_5783, Rc_11301, Rc_10933, Rc_5763, Rc_8731* & *Rc_11752* Group 3, 4-8dpi; *Rc_11163* Group 4, 10-21dpi

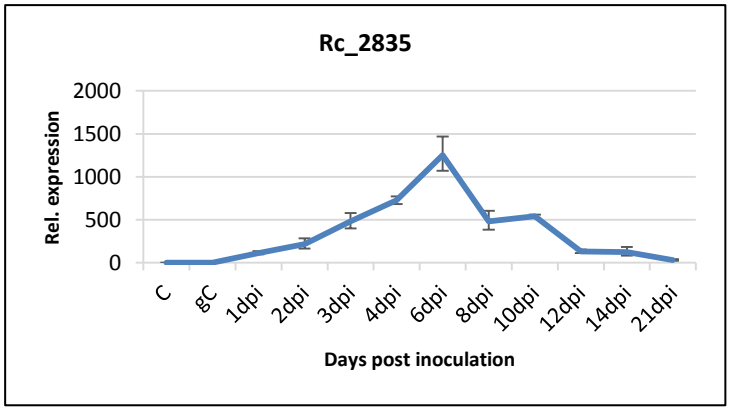


Figure 3.4: Relative transcript abundance of *R. commune* candidate effector *Rc_2835* during infection of barley with *R. commune* strain L2A normalised against *R. commune* endogenous control Actin. Error bars indicate confidence intervals of the 3 technical repetitions.

3.2.4.2 Analysis of expression of selected candidate effectors during infection *in planta* and culture media using two different *R. commune* strains

Three novel candidates, *Rc_10934, Rc_2091* and *Rc_2835,* were selected for further analysis as they were some of the highest expressed during infection and showed a similar expression profile to that of the NIPs (Kirsten *et al*., 2012). In addition, *Rc_10934 & Rc_2091* were identified in germinated conidia and early interaction transcriptomes (unpublished data, Avrova).

The selected candidates shared the same expression profile, inclining from 1 dpi with highest expression at 6 dpi before declining at 8 dpi and subsequently at 10 dpi. The increase in transcript abundance between the three candidates varied considerably. *Rc_2835* showed the highest level of upregulation, reaching a substantial 1200-fold increase at 6 dpi compared to its level in conidia. At the peak of its expression *Rc_2835* transcript was almost as abundant as actin (Figure 3.5 C). Both *Rc_10934* and *Rc2091* were highly upregulated during barley infection compared to their levels in conidia, with a 150 fold and 25-fold increase respectively (Figure 3.5 A-B). At the peak of their expression *Rc_10934* and *Rc2091* transcripts were 1.5 and 5.5 times as abundant as actin respectively (Figure 3.5 A-B).

The general conception for effector gene expression is that transcripts are highly abundant during infection of their host (Koeck *et al*., 2011; Alfano., 2009; Kamper *et al*., 2006). Therefore, it may be reasonable to assume that there is a distinction between genes that are required for vegetative growth and those that are required for pathogenesis. To distinguish between candidates that are important during infection but not expressed during vegetative growth, *R. commune* was grown in PDB for a period of 8 days. Fungal mycelia were collected over different time points and selected genes

were analysed for expression (Figure 3.5 D-F). Both *Rc_2091* and *Rc_10934* were not upregulated at any stage of growth in liquid PDB medium and showed similar abundance of 0.15 and 0.2 of the level of actin in conidia with much lower levels in liquid medium. By contrast, the increase in transcript abundance of *Rc_2835* was evident from 2 dpi until 6 dpi reaching a maximum of 35-fold compared to transcript abundance in conidia. However, it was clear from the data that the transcript levels of the selected candidates were specifically upregulated to a much higher level during infection, suggesting a role in pathogenesis.

A second separate infection time course containing three biological repetitions was used to further validate the results for the effector candidates. In this instance a different *R. commune* strain was used for *in planta* and *in vitro* infection, which were also conducted separately. Confocal microscopy was used to observe the fungus and its progression throughout the infection process. Images taken during infection of the GFP expressing *R. commune* strain 214-GFP reveal germinating conidia at 1dpi, penetration of the barley leaf cuticle at 3dpi, epidermal colonisation at 4-8dpi, and extensive colonisation of the apoplast with proliferation of mycelium out from the inoculation zone, eventually leading to plant cell collapse (Figure 3.7). However, the infection experiment was not run in parallel with the 214-GFP experiment.

Despite *Rc_2091* having similar levels of expression in both L2A and 214-GFP infection time courses, candidate transcript abundance for *Rc_10934* and *Rc_2835* was half of the levels identified in L2A infected samples (Figures 3.5 & 3.6 A&E). . The timing of expression for *Rc_2835* during 214-GFP infection began earlier, showed lower expression levels at 2 and 4 dpi and continued further to 8dpi (Figures 3.5 & 3.6 C). Analysing the expression profiles in medium between both strains it was evident that again both *Rc_10934* and *Rc_2091* transcript abundance was extremely low (Figure

3.6 D & E) and for *Rc_2835* the expression level was very similar to that seen in L2A strain (Figure 3.6 F). The results confirm the expression of candidate effectors in two different *R. commune* strains suggesting their importance for the pathogen. Additionally, L2A is a more aggressive strain in comparison to 214-GFP (Gamble, unplublished data) and therefore expression timing and levels would be expected. Again, results revealed that all selected genes are upregulated during infection.

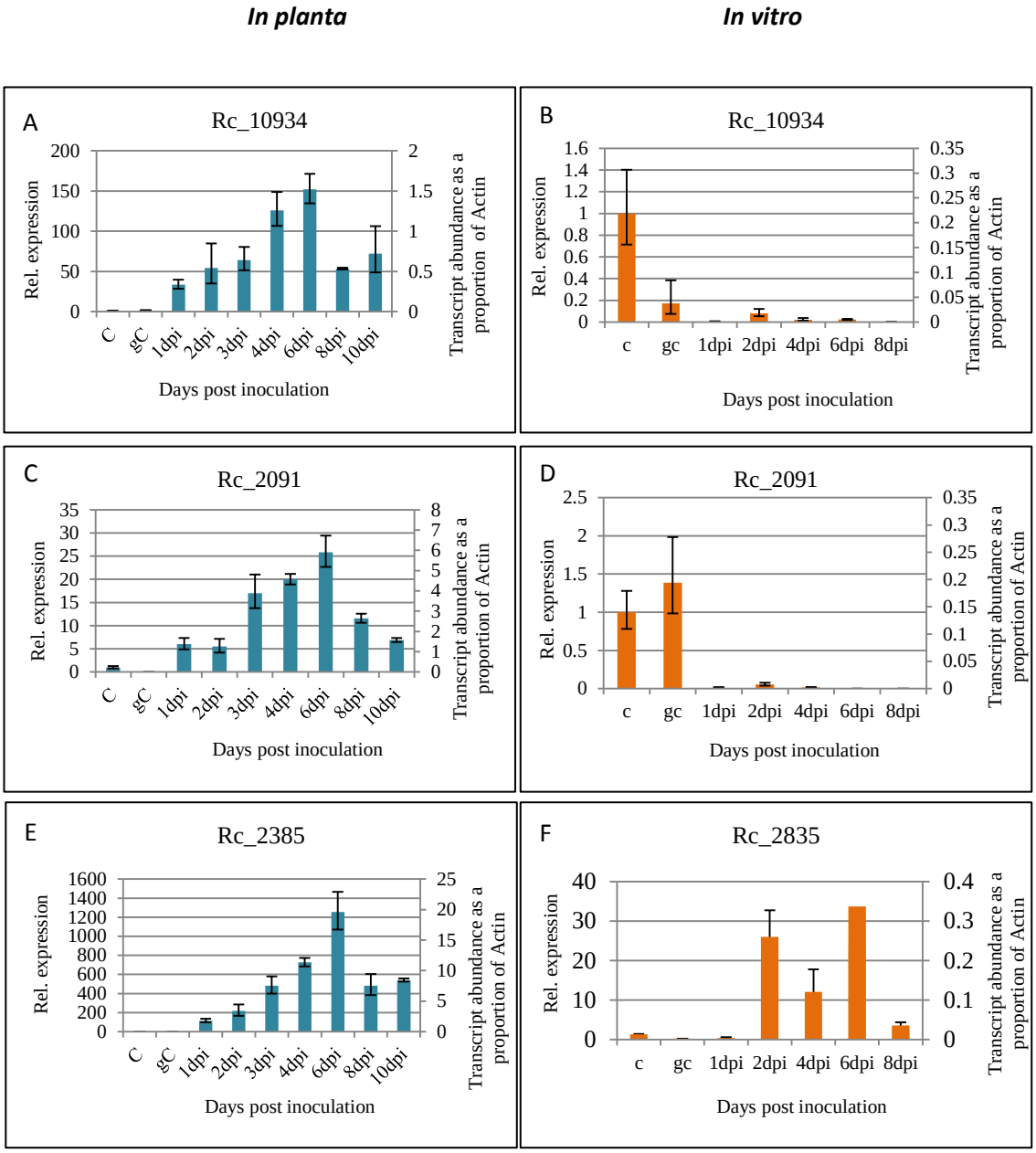
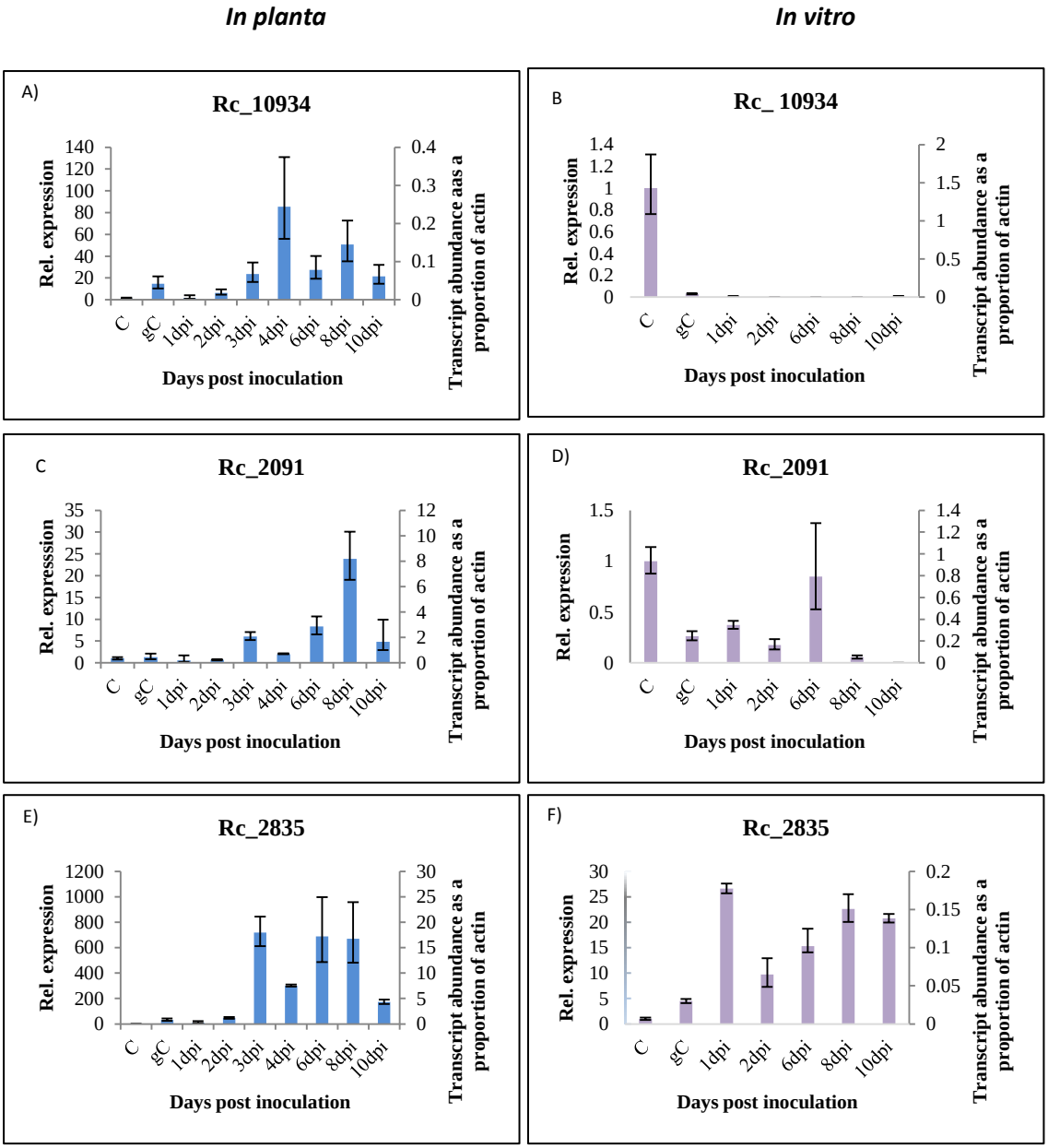


Figure 3.5: A,C&E) Relative expression of selected *R. commune* genes during infection of susceptible barley cultivar Optic with *R. commune* strain L2A; B,D&F) Relative expression of selected genes in *R. commune* strain L2A during growth in liquid PDB medium. Error bars indicate the standard error for the average of three technical repetitions.



Figures 3.6 A,C&E) Relative expression of selected genes during infection of susceptible barley cultivar Optic with *R. commune* strain 214-GFP; Error bars indicate the standard error for 3 biological replicates. B, D&F) Relative expression of selected genes in liquid PDB medium. Expression of all genes is normalised against their level in conidia using *R. commune* actin as the endogenous control. Error bars indicate the standard error for 2 biological replications.

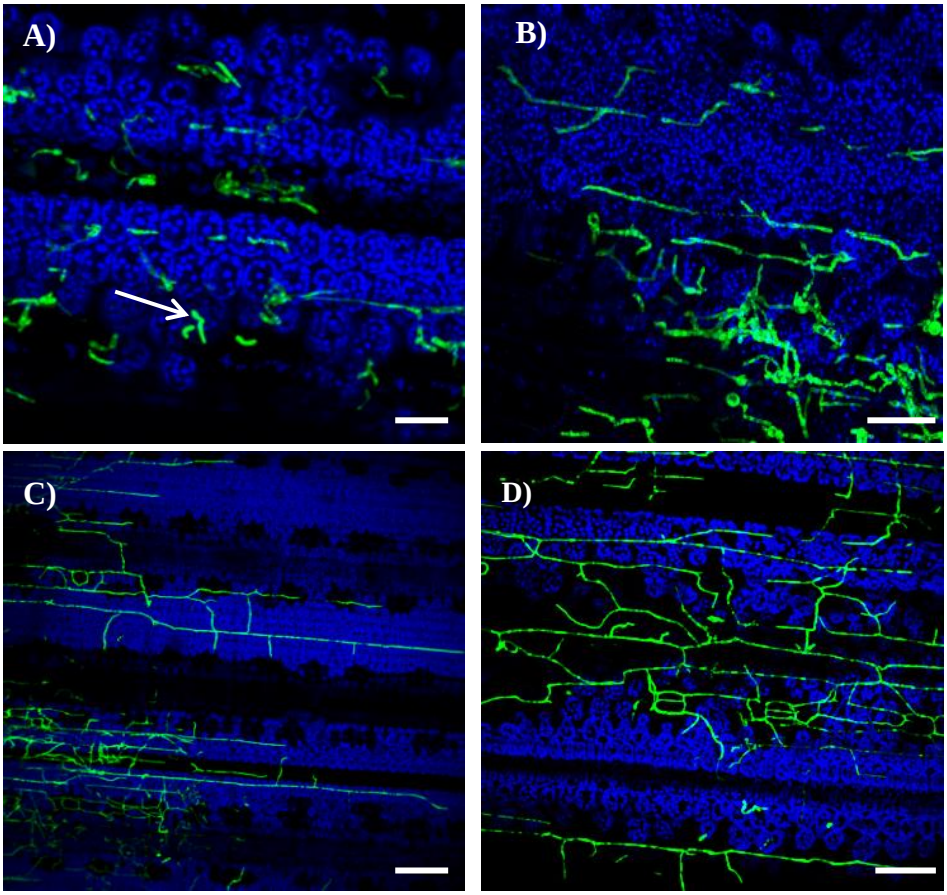


Figure 3.7: Confocal images of the infection progress following inoculation of susceptible barley cultivar Optic with *R. commune* strain 214-GFP excitation of 488 nm and emission collection of 500-530 nm. The autofluorescence signal from plant chlorophyll was collected with an emission range of 650-700 nm. A) 1 dpi germinating conidia, B) 3 dpi penetration of the cuticle and onset of epidermal tissue colonisation, C) 6 dpi colonisation of the epidermal tissue and growth out with the inoculum spot, D) 10 dpi extensive colonisation of the apoplast. Scale bars A, C, D 50μm and B 100μm. Images are representative of five biological repetitions.

3.2.5 *R. commune* transformation to elucidate gene function

Targeted gene disruption was used to functionally characterise the candidate effectors selected based on their expression profiles and to determine if their function is essential for pathogenesis. The first approach to integrate the cassette into the DNA of *R. commune* strain L2A was pursued via transformation of protoplasts. This method provided nearly 100% efficiency rate of integration but not at the specific gene location. After some attempts to obtain a knockout it became evident that a large number of transformations would need to be performed, so another strategy was adopted. Electroporation of *R. commune* conidia was used to deliver the deletion cassette into the fungal cell. In this instance a different strain L73A was used as isolate L2A had lost pathogenicity. Conidia electroporated without any cassette was used as a control to assess viability of *R. commune* conidia after electroporation and showed normal growth with colonies appearing around 8-10 days, indicating the procedure had no effect on the germination of the fungal conidia and the growth of the mycelia.

All 3 candidates were selected in the attempt to knock out the gene of interest and obtain a phenotype. Each candidate knockout was attempted three times and resulted in the creation of between 60 to 100 transformants for each attempt. Transformed colonies began to form on the selective media around 10-12 days revealing the ability of the electroporated conidia to form mycelia and sporulate in the presence of the antibiotic, suggesting the presence of the resistance marker. This was further confirmed using specific primers to determine the presence of the hygromycin resistance gene (Figure 3.9 A & B). Sequencing of the deletion cassette provided further evidence to confirm the successful integration into the fungal genome. Again, this approach resulted in a very high level of efficiency of cassette integration.

The next step was to investigate if the gene of interest had been replaced with the deletion cassette. The 5' UTR forward primer (G1) was used with a reverse primer designed to amplify from within the ORF (G2) of the candidate effector gene to check if the wild type gene was still present (Figure 3.8 A). The amplification of the region downstream from the 5' UTR region (G1 primer) in combination with a region of the hygromycin resistance gene (Hyg R) to confirm gene replacement. Lastly amplification of the targeted area from upstream and downstream regions of the flanking regions was utilised to determine size differentiation between the wild type gene and mutant gene insertion (results not shown). A typical result of genotyping of the transformants is detailed in Figure 3.8 B. Successful amplification of the Hygromycin resistance gene (Lane 2), amplification of the wild type gene (Lane 3) and no amplification of the mutant gene (Lane 4). Amplification of actin was used as a loading control. No candidate effector genes were knocked out and time restrictions limited any further continuation of the approach.

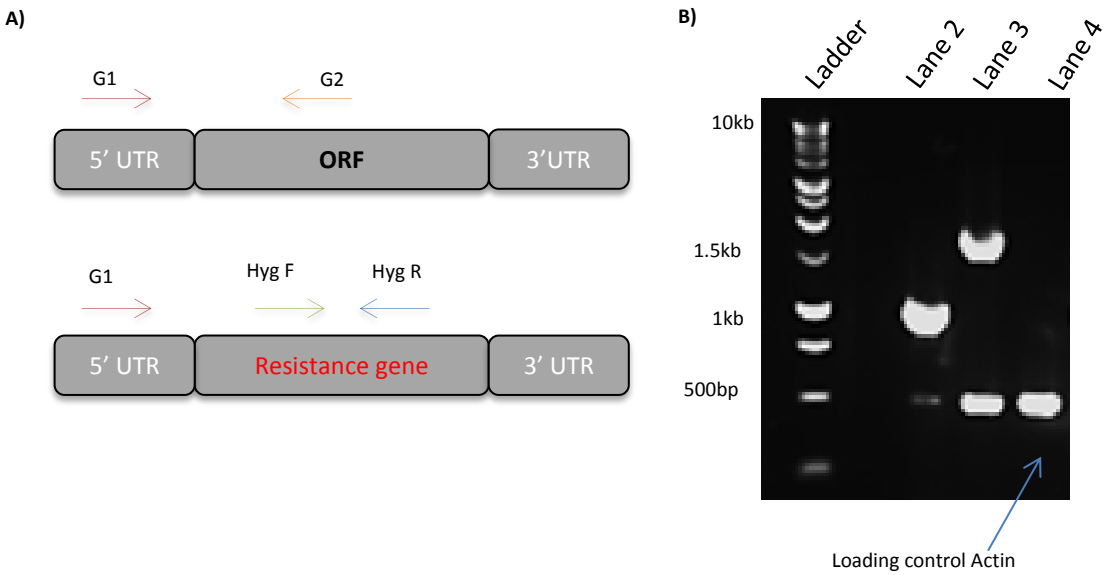


Figure 3.8: Genotyping of *R. commune* transformants. A) Primer locations used for genotyping strategy to determine targeted gene disruption and hygromycin resistance gene insert. Red_5' UTR G1. Orange- G2, wild type ORF reverse. Green hygromycin forward, Blue hygromycin reverse. B) 1.5 % agarose gel loaded with the 1kb ladder (Lane 1) and PCR products produced usin Hygromycin F&R primers (Lane 2), G1 &G2 primers – amplification of wild type (Lane 3) and No amplification with G1and HYG R primers (Lane 4)

3.2.6 Analysis of the apoplastic proteome during infection reveals the presence of potential effectors

To finalise the research of effector identification, apoplastic fluid extracted from the barley leaves inoculated with *R. commune* strain L73A was analysed to confirm the presence of candidate effector proteins during a susceptible interaction. Two time points were selected, 4 dpi - which represents the initial colonisation of the apoplast and 7 dpi in which growth of the fungus would be well developed. In addition, the majority of candidate effectors were expressed within this timeframe.

3.2.6.1Enzyme contamination assay

To test the level of cellular contamination of apoplastic fluid, the activity of glucose-6-phosphate dehydrogenase (G6PDH) was analysed. Homogenised leaf material infected with *R. commune* and non-infected were used as a positive control. The enzyme activity was measured as the rate of increase in absorbance at 340 nm (ΔA340) per min. Apoplastic extract from non-infected plant resulted in 2.89% contamination in comparison to enzyme activity of whole tissue. Infected barley leaves showed a higher level of the enzyme, just under a 1% increase. This is likely due to the potential breakdown of plant cells during infection. The results are summarised in table 3.2.

Table 3.2: Determination of the contamination of the apoplastic extracts (means ± se, n = 3) of G6PDH activity of apoplast extract

	Whole tissue	Apoplast (%)
Non-infected barley leaf	0.96 ± 0.13	2.89%
Infected barley leaf	1.35 ± 0.117	3.79 %

3.2.6.2 *R. commune* proteins identified in the apoplast

Four and twenty *R. commune* proteins were identified at four and at seven dpi respectively (table 3.3). Sequence analysis to identify signal peptides for the secretion into the apoplast were predicted using SignalP and TargetP. All the proteins identified were predicted to be part of the secretory pathway except from RCO7_10779 which was predicted to be localised to the mitochondria (Table 3.3). Using InterPro (https://www.ebi.ac.uk/interpro/), a functional protein analysis online resource, it was possible to assign biological processes to a large proportion of the identified fungal proteins, revealing roles in nutrient acquisition, stress and defence against plant immunity (Table 3.4, Figure 3.9). In addition, numerous plant proteins were also identified during the compatible interaction but were not included in this research but are detailed in the appendix (Table 9.48).

Plant cell wall degrading enzymes (CWDEs) were the most highly abundant proteins in the apoplast during infection (Figure 3.9). Enzymes involved in the breakdown of xylan (*Rc_07824*), lignin (*Rc_07699*), pectin(*Rc_03266*) and cellulose (*Rc_00972*) were identified. This was not surprising as CWDEs play a significant role in pathogenesis with the ability to depolymerize the main structural polysaccharide components of the plant cell wall (Kubicek *et al*., 2014). Furthermore, amongst the most abundant a putative glucose-methanol-choline (GMC) oxidoreductase was expressed which has been suggested to be a lignocellulose acting enzyme (Couturier *et al*., 2015). Two different types of proteases were identified, a serine type carboxypeptidase and a subtilisin like protease. In many cases proteases are considered to be virulence factors of many pathogenic species (Hoge *et al.*, 2010).

Similar to many plant pathogens, *R. commune* secretes a probable catalase peroxidase at both 4 and 7 dpi with a high up regulation of the protein at the latter time point of infection. The importance of catalase peroxidases to circumvent the effects of plant defence have been highlighted in numerous studies (Zámocký *et al*., 2009). Catalase-peroxidase proteins are known to detoxify the products of the oxidative burst in the apoplast upon the triggering of plant immunity. Tanabe *et al.* (2011) demonstrated that one of the three catalase peroxidase genes identified in *Z. tritici* plays an important role in pathogenicity. MgDCat-1 is also upregulated during infection and most abundant at 8 dpi.

Although none of the candidate effectors identified in the bioinformatics pipeline described in this chapter were detected in apoplastic fluid from barley leaves infected with *R. commune*, proteomics analysis identified four other potential effectors. This included two proteins which had been previously highlighted as candidate effectors but

not in the original panel, *Rc07_03591* which showed homology to an effector like protein from powdery mildew, *B. graminis f. sp. hordei*., and *Rc07_02334* a hypothetical fungal protein from the anamorphic fungus *Glarea lozoyensis*. The best BLASTp hit for *RC07_10338* matched EC13 protein from anthracnose leaf spot which has been shown to be expressed during the establishment of biotrophic hyphae (Kleemann *et al*., 2008). Lastly, protein *Rc_02661* which contained three LysM domains was also identified. Interestingly LysM domain proteins have been well characterised in several plant pathogens and shown to play a fundamental role in fungal pathogenesis (Kombrink, 2013).

Table 3.3 Signal peptide and localisation predictions of *Rhynchosporium commune* proteins identified in the apoplast

Protein Id	Amino acid length	TargetP localisation	SignalP position
RCO7_03591	196	s	18/19
RCO7_11633	789	s	23/24
RCO7_07041	775	s	16/17
RCO7_07699	606	s	29/30
RCO7_10338	170	s	24/25
RCO7_09037	437	s	24/25
RCO7_04918	176	s	18/19
RCO7_02661	185	s	16/17
RCO7_07332	462	s	33/34
RCO7_10779	468	m	-
RCO7_00972	466	s	19/20
RCO7_10679	439	s	24/25
RCO7_07191	448	s	19/20
RCO7_03266	350	s	16/17
RCO7_02334	181	s	18/19
RCO7_01317	326	s	19/20
RCO7_07824	328	s	20/21
RCO7_07974	638	s	19/20
RCO7_11478	437	s	18/19
RCO7_03061	**3248**	**s**	**45/46**

Table 3.4 MS Log values of the intensity of identified apoplastic proteins, BLASTp hit and predicted biological function. Intensity values are associated with peptide and m/s values.

Protein Id	Intensity Infected 4 dpi	Intensity Infected 7 dpi	Best BLASTp hit	Species	Biological function
RCO7_03591	NaN	28.7554	CELP0025 Effector like protein	*Blumeria graminis f. sp. hordei*	unknown
RCO7_11633	20.6542	25.9635	subtilisin-like protease	*Colletotrichum incanum*	proteolysis
RCO7_07041	17.6621	25.1265	catalase/peroxidase HPI	*Phialocephala scopiformis*	response to oxidative stress
RCO7_07699	NaN	24.7095	putative glucose-methanol-choline oxidoreductase	*Diaporthe ampelina*	oxidation-reduction process
RCO7_10338	NaN	24.0785	EC13 protein	*Colletotrichum higginsianum*	unknown
RCO7_09037	NaN	23.6316	serine-type carboxypeptidase F	*Aspergillus udagawae*	proteolysis
RCO7_04918	19.4859	22.598	putative glycosyl hydrolase family 43	*Colletotrichum sublineola*	carbohydrate metabolic process
RCO7_02661	NaN	22.6916	putative cell wall-associated hydrolase	*Marssonina brunnea f. sp. 'multigermtubi'*	unknown
RCO7_07332	NaN	21.7159	carbohydrate-binding module family 6 protein	*Bipolaris zeicola 26-R-13*	unknown
RCO7_10779	NaN	21.4069	GPI-anchored cell wall beta-endoglucanase	*Marssonina brunnea f. sp. 'multigermtubi'*	carbohydrate metabolic process
RCO7_00972	NaN	21.1876	Glycosyl hydrolase family 6, cellulase	*Glarea lozoyensis ATCC 20868*	carbohydrate metabolic process
RCO7_10679	NaN	20.6017	putative exopolygalacturonase B	*Marssonina brunnea f. sp. 'multigermtubi'*	carbohydrate metabolic process
RCO7_07191	NaN	20.5373	putative glycoside hydrolase family 7 protein	*Botrytis cinerea BcDW1*	carbohydrate metabolic process
RCO7_03266	NaN	20.3824	pectin methyl esterase	*Marssonina brunnea f. sp. 'multigermtubi'*	cell wall modification
RCO7_02334	NaN	19.7302	hypothetical protein GLAREA_02918	*Glarea lozoyensis ATCC 20868*	unknown
RCO7_01317	NaN	19.5645	hypothetical protein MBM_04331	*Marssonina brunnea f. sp. 'multigermtubi'*	unknown
RCO7_07824	NaN	19.5446	putative endo-1,4-beta-xylanase B precursor	*Pyrenochaeta sp. DS3sAY3a*	carbohydrate metabolic process
RCO7_07974	NaN	18.6409	hypothetical protein V499_03635	*Pseudogymnoascus sp. VKM F-103*	unknown
RCO7_11478	NaN	17.5457	Zn-dependent exopeptidase	*Glarea lozoyensis ATCC 20868*	proteolysis
RCO7_03061	17.499	NaN	fermentation associated protein	*Marssonina brunnea f. sp. 'multigermtubi'*	fermentation

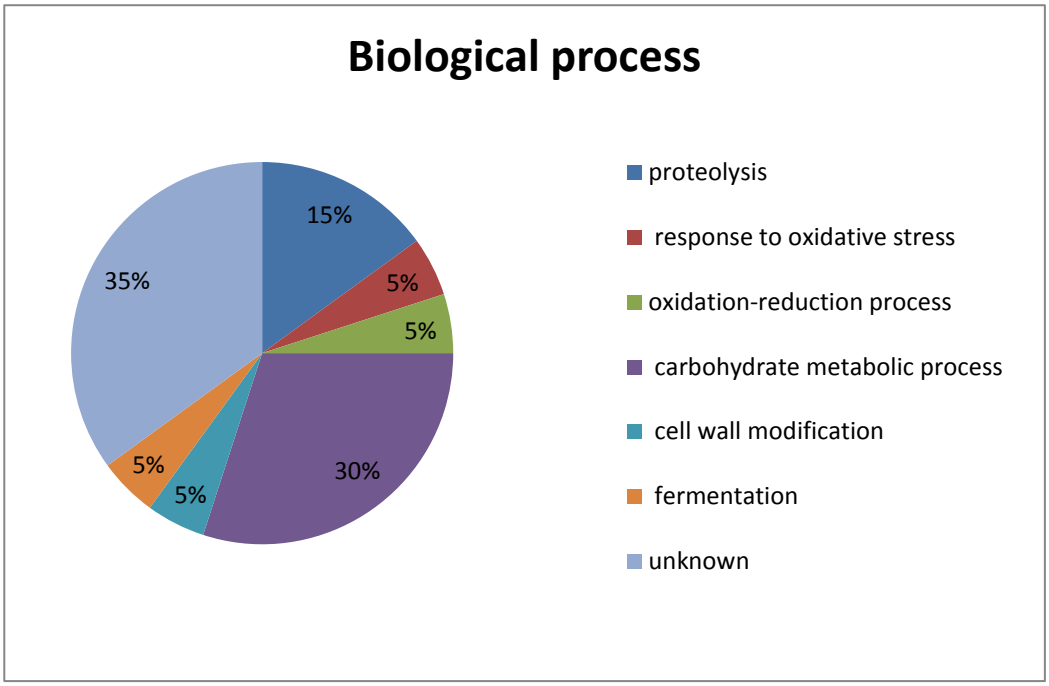


Figure 3.9: Percentage of *Rhynchosporium commune* proteins identified in susceptible barley cultivar Optic apoplastic fluid within each biological process category

3.3 Discussion

Using common effector characteristics as a baseline resulted in the identification of some interesting candidate effectors from *R. commune*. In addition, the complementary proteomic analysis of apoplastic fluid from infected barley leaves not only identified some interesting candidate effectors but revealed some fundamental molecules involved during the infection of barley.

The BLAST search was an efficient alternative to reveal any conservation at the sequence level as no effector-specific motifs have been identified for *R. commune* so far. Results from the BLAST search indicated that the majority of the significant matches to candidates were novel, identified as hypothetical proteins with no homology to other effectors. This suggests that they are likely to be novel potential effectors and confirms that they are species specific. But it is possible that due to many effectors still to be identified, the information of similar effector sequences is not available yet.

On the other hand, the fact that some small cysteine rich proteins may not have effector function, there is a risk for the predicted candidates to be imposters of the effector title and hence further refinement is often required (Pritchard & Broadhurst, 2014). However, large proportions of effectors that have been identified already have no obvious similarity to each other or effectors from other species in the databases and tend not to be lineage specific (Kamoun *et al*., 2009; Rovenich *et al*., 2014). In fact, *R. commune* NIPs do not share any sequence similarity to each other, nor to any other known effectors. In some instances, plant pathogen effectors have been previously identified that do not adhere to all of the general paradigm for effector identification. It may be assumed that high cysteine content is required for

effectors that are secreted into the apoplast to obtain stability. However, apoplastic effectors such as ECP6, ECP2 (*C. fulvum*), Pep1 and Pit2 (*U. maydis*) all contain low percentage of cysteines (Sperschneider *et al*., 2015).

Transcription profiling identified candidates which are highly up-regulated during infection suggesting a role in pathogenesis. With many of the candidates being expressed at the biotrophic stage of infection the fungus would be required, like most biotrophs to obtain nutrients from the host (Koeck *et al*., 2011). It is possible that the candidate effectors identified may have a role in nutrient acquisition similar to the proposed function of the NIP1 & NIP3 (Kirsten *et al*., 2012). Additionally, effectors highly abundant during this time may serve to supress host defence mechanisms (Djamei *et al*., 2011). However, the inability to successfully disrupt any of the selected candidates resulted in inability to determine the function of the effector gene or its importance for pathogenesis.

There are many factors that may influence targeted gene disruption including the genomic position of the target gene, the transformation method and the length of the homologous sequence (Kück & Hoff., 2010). Although different strategies were used in this research the efficiency of integration events can be hastened by non-homologous end joining (NHEJ), an alternative DNA repair pathway (Valencia *et al*., 2001). One method for fungi with a low frequency of homologous recombination is to decrease the expression of genes involved in non-homologous end-joining (Ninomiya *et al*., 2004; Weld *et al*., 2006). This approach was already being tested within the research group. However, a more convenient approach may have been to increase the expression of genes involved in homologous recombination (Natume *et al*., 2004; Weld *et al*., 2006)

Gene silencing can also be used to reduce the expression of the gene and determine whether the phenotype is altered. Transient gene silencing using delivery of dsRNA into *P. infestans* protoplasts to trigger silencing, was used to identify the requirement of a novel haustorium-specific membrane protein for infection of potato (Avrova *et al*., 2008; Whisson *et al*., 2005). Furthermore, the overexpression of candidate effectors *in vitro* (Bhadauria *et al*., 2013) or transienft overexpression *in planta* (Bos *et al*., 2010) can provide information on effector function.

Despite the lack of any functional characterisation, the identification of additional candidate effectors in the apoplast during infection was an important discovery and provided confirmation of their actual presence. However, as previously documented there are limitations of protein detection from apoplastic fluid extraction (O'Leary *et al*., 2014). The main drawbacks of the procedure include the lack of capture of all molecules present, and that the procedure itself may affect the amount of proteins identified. Only two time points were selected and therefore many proteins involved in the interaction may have been missed and the results will tend to be biased towards the chosen timepoints. However, the timing of the sampling corresponds with the vast majority of expressed proteins *in planta*.

Nevertheless, proteins involved in pathogenesis were identified. The high abundance of the cell wall degrading enzymes (CWDEs) highlights the ability of *R. commune* to degrade host cell walls to maximise the nutrient availability (Zhao *et al*., 2013). Plant pathogenic fungi have been shown to possess the highest number of CWDEs in general (Zhao *et al*., 2013). In addition, the presence of particular CWDEs have been shown to reflect host preference among plant pathogenic fungi (King *et al*., 2011). Targeting of plant defence mechanisms was also evident with the identification of *R. commune* proteases that have been shown to modify or

degrade pathogenesis-related proteins, including plant chitinases (Chandrasekaran *et al*., 2016).

Overall, approaches used in this work have identified candidate effectors and helped to prioritise them for further analysis. The candidates are predicted to contain all the common characteristics of apoplastic effectors with a very high transcript abundance for the three selected candidates during the biotrophic stage of interaction with barley. Furthermore, another four potential effectors have now been confirmed to be present in the apoplast during the actual infection. Therefore, this research has presented some interesting candidates for future work. The *in vivo R. commune and* barley apoplast secretome databases that have been generated in this research serves as a valuable resource in the analysis of *R. commune*-barley interaction giving specific insight into *R. commune* and barley proteins present during an interaction.

A different strategy adopted for gene disruption and the identification of further effector characteristics will aid in a faster screening approach for characterisation of *R. commune* effectors.

CHAPTER 4

4. The identification and characterisation of a family of LysM-domain proteins

4.1 Introduction

Chitin, a polymer of N-Acetyl-D-glucosamine, is an essential structural component of fungal cell walls and an important factor of fungal pathogenicity (El Gueddari *et al*., 2002; Vega & Kalkum, 2011). In *R. commune* cell wall, chitin was shown to be predominantly present in the inner layer, accounting for 7 % of its polysaccharide composition, with only traces present in the outer cell wall (Pettolino *et al*., 2009). Fragments of chitin are likely to be released from the fungal cell wall as *R. commune* continues to extend throughout the apoplast during infection. Plants lack chitin and subsequently, this polysaccharide is recognised as a PAMP, resulting in the activation of a plant immune response (Jones & Dangl, 2006). It has been shown that fungal chitin perception by plant pathogen recognition receptors (PRRs) results in a MAPK cascade signalling pathway activating the defence network against fungal pathogens (Wan *et al*., 2008).

Plants also secrete enzymatic chitinases into the apoplast that degrade chitin fragments and attack the fungal cell wall (Zamir *et al*., 1993). To evade the activated host immune response, pathogens need to adopt mechanisms to conceal the fragments of chitin and protect their cell wall. This is specifically important for apoplastic pathogens as they are limited to this compartment until they complete their lifecycle (Stotz *et al*., 2014).

It is well documented that many apoplastic pathogens secrete LysM-domain effectors to prevent the recognition and/or protect the fungus from plant defence response (Kombrink & Thomma, 2013). The LysM motif is a very well characterised domain that has been shown to function in the binding of different polysaccharides including chitin and its derivatives (Buist *et al*., 2008). It typically contains around 40 – 65 amino acid residues and has a βααβ secondary structure (Batemann & Bycroft, 2000; Mesnage *et al.,* 2014).

The LysM domains are prevalent in many other organisms and were first identified in enzymes that degraded bacterial cell walls (Garvey *et al*., 1986; Buist *et al*., 2008). The motifs have been identified in a wide range of proteins including secreted proteins, membrane proteins and cell wall anchored proteins (Guste *et al*., 2012). Importantly, they have been shown to play a fundamental role in the infection process of apoplastic pathogens through their ability to bind chitin and prevent host immune responses to the pathogen infection. For instance, the best characterised LysM-domain effector was found in the tomato pathogen *C. fulvum* which expresses two LysM-domain effectors. Ecp6 is a small protein containing three LysM domains and has been shown to bind chitin (Bolton *et al*., 2008; De jonge *et al*., 2010; Sanchez Valley *et al*., 2013). A second protein Avr4 has a chitin binding domain and functions by binding to the fungal cell wall to provide protection from plant chitinases (van den Burg *et al*., 2006). Avr4 has been shown to bind longer chains of chitin and can therefore bind to the outer cell wall of the fungus to prevent the degrading capabilities of plant chitinases. Avr4 can also be recognised by the corresponding tomato resistance gene Cf4 but can evade this recognition (van Esse *et al*., 2007). Two cysteine residues are changed to tyrosine in

isoforms that are no longer recognised. However, impressively it still manages to carry out its function of chitin binding, suggesting that the binding of the PAMP chitin is of utmost importance for pathogen virulence.

Since their discovery many other chitin binding effectors have been identified, including Mg1LysM, MgxLysM and Mg3LysM from the wheat pathogen *Zymoseptoria tritici* (previously known as *M. graminicola*) (Marshall *et al*., 2011). Mg1LysM and Mg3LysM are highly expressed during infection and were shown to bind chitin, but not chitosan, xylan or cellulose (Marshall *et al*., 2011). A knock out of both of the genes revealed that Mg1LysM is required for full pathogenicity. In the rice blast fungus *Magnaporthe grisea* Slp1, a LysM effector protein contains two LysM domains and also binds to chito-oligosaccharides. The expression of this protein suppresses chitin-induced plant immune responses (Mentlak *et al*., 2012). More recently, the extracellular LysM domain proteins (ChEIp1 and ChEIp2) from *Colletotrichum higginsianum* that causes anthracnose on *Brassicae*, have been shown to play dual roles in appressorial function and suppression of chitin-triggered plant immunity (Takahara *et al*., 2016).

It is clear that the ability to conceal chitin and protect the fungal cell wall within the apoplast is an effective strategy and there has been much research dedicated to the understanding of the LysM fungal protein effectors. Hence, it was of interest to investigate the presence of genes coding for potential LysM-domain effectors in the *R. commune* genome.

4.2 Results

4.2.1 A large family of LysM domain genes identified in the *R. commune* genome

As a result of identifying *Rc_02261* now renamed as *RcLysM3*, during infection of the plant apoplast in the previous chapter, the *R. commune* genome database was searched for the presence of other LysM domain proteins, using the motif as a reference sequence. This led to identification of a family of nine genes coding for LysM domain containing proteins (Figure 4.1), which is an even larger family than the significantly expanded LysM effector family of six to seven members in the soil borne fungal plant pathogen, *Verticillium dahlia* (de Sain & Rep, 2015). Sequence analysis was performed on each of the nine sequences as in the previous chapter. No signal peptides were identified for RcLysM5, RcLysM6 and RcLysM7 but they were predicted to be non-cytoplasmic, with RcLysM5 and RcLysM6 predicted to be secreted via an unconventional secretory pathway (Figure 4.1).

Interestingly the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) identified a predicted transmembrane domain located in the middle of RcLysM7 protein (Figure 4.1). The three top BLASTp hits were analysed for the presence of TM domain. All three showed similar structural architectures – a cytoplasmic domain coupled with a TM domain and the presence of one or more LysM domains. This type of proteins has not been identified as a fungal effector, but has been found in plant chitin receptors which are bound to the plasma membrane and function to bind chitin (Eckardt, 2008).

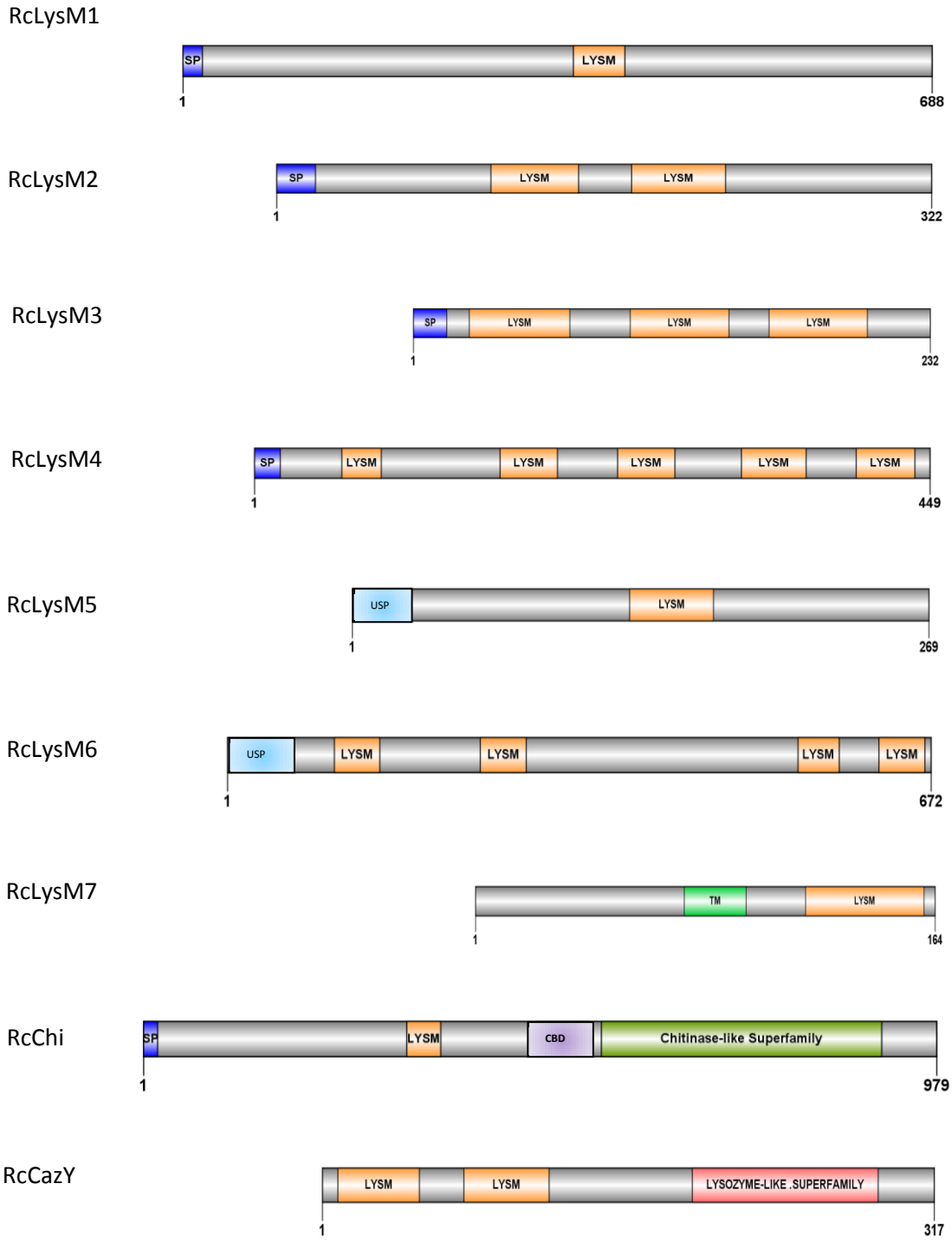


Figure 4.1: Schematic amino acid sequence diagrams of LysM domain proteins identified in *Rhynchosporium commune* (not drawn to scale), LysM domains are highlighted in orange. SP, signal peptide, in blue and unconventional signal peptide in light blue. TM, transmembrane domain, in green. CBD, chitin binding domain, in purple. Chitinase-like superfamily domain in green and Lysozyme like superfamily domain in red.

BLASTp searches revealed the presence of varying numbers of LysM domains within the sequences, detailed in Figure 4.1. Four LysM domain proteins identified, RcLysM1, RcLysM5, RcLysM7 and RcChi, contained one LysM domain, while RcLysM2 and RcCAZy contained two LysM domains (Figure 4.1). Similar to the well characterised Ecp6 effector from *C. fulvum*, RcLysM3 contained three LysM domains, whereas RcLysM4 and RcLysM6 contain five and four domains respectively (Figure 4.1). One of the LysM domains identified in RcLysM4 was smaller than expected of the LysM domain containing 38 amino acids in comparison to the documented minimum amount of forty amino acids (Batemann & Bycroft, 2000; Mesnage *et al*., 2014). A Chitin binding domain (CBD) was also revealed alongside a chitinase family domain in RcChi (Figure 4.1). Fungal chitinases have been shown to have multiple functions including nutrition, fungal development and in some cases mycoparatism (Hamid *et al*., 2013).

Another enzymatic type of domain known as a lysozyme-like domain was identified in RcCAZy (Figure 4.1). Lysozymes have mainly been characterised as a broad group of enzymes that degrade bacterial cell walls through hydrolysis of 1,4-beta-linkages (Salazar & Asenjo., 2007). The top BLASTp hit for this protein is a hypothetical protein from *M. brunnea*, which shared sequence similarity at the points of the two LysM domains. A similar lysozyme-like domain was also found in a protein from *Pochonia chlamydosporia* a nematophagous fungi (Larriba *et al*., 2014). The protein is classified as a glycosyl hydrolase which assists in the breakdown of complex carbohydrates found in many fungal species (Goedegebuur *et al*., 2002). Unlike RcChi, the RcCAZy sequence does not contain any signal peptide. RcLysM1 shared homology to a LysM containing protein from

Colletotrichum graminicola. The best BLASTp hit for both RcLysM2 and RcLysM3 was to a cell wall hydrolase from *Marssonina brunnea.* Results of sequence analysis and BLASTp top hits can be found in Table 4.1.

Table 4.1: Amino acid sequence analysis of *R. commune* LysM domain proteins.

Sequence Id	Protein length, aa	Number of Cysteines	Localisation	Top BLASTp match	Species	Accession #	E value
RcLysM1	688	30	s	LysM domain-containing protein	Colletotrichum graminicola	XP008092567.1	0
RcLysM2	332	7	s	putative cell wall-associated hydrolase	*Marssonina brunnea*	XP_007296068.1	3.0E-32
RcLysM3	232	8	s	putative cell wall-associated hydrolase	*Marssonina brunnea*	XP_007296068.1	4.0E-34
RcLysM4	449	20	s	LysM domain-containing protein	*Colletotrichum tofieldiae*	KZL71376.1	0
RcLysM5	269	10	other	hypothetical protein	*Phialocephala scopiformis*	KUJ21079.1	9.0E-146
RcLysM6	672	35	other	LysM domain-containing protein	*Colletotrichum graminicola*	XP_008100462.1	3.0E-139
RcLysM7	164	4	other	carbohydrate-binding module family	*Glonium stellatum*	OCL02051.1	1.0E-43
RcCAZy	317	4	other	hypothetical protein	*Marssonina brunnea*	XP_007294669.1	1.0E-160
RcChi	979	34	s	glycosyl hydrolase family 18	*Colletotrichum incanum*	KZL82818.1	0.0E+00

4.2.2 Phylogenetic analysis of LysM domains from members of *R. commune* LysM domain protein family

Using PRALINE, the online mutliple sequence alignment program (http://www.ibi.vu.nl/programs/pralinewww/), the approximatley 50-residue amino acid sequence from each LysM domain found in the *R. commune* proteins identified from BLASTp and *C. fulvum* Ecp6 were aligned to investigate sequence similarities (Figure 4.2). The first two thirds of the domains showed a strong similarity in contrast to the remaining third of the domains. This highlights that LysM domains share homology but differences are present which is to be expected due to LysM domains variable functions. To determine any relatedness, a phylogram was also generated (http://www.phylogeny.fr/.) All three LysM domains from RcLysM3 and both LysM domains from RcLysM2 were grouped with the LysM domains form Ecp6 (Figure 4.3). In contrast, the remaining *R. commune* LysM domains were not as closely related. The domains from RcLysM1, RcLysM4, RclysM5 and RcLysM6 clustered together (Figure 4.3). RcCAZy LysM domains were more closely related to the first group rather than the latter (Figure 4.3).

Four conserved cysteines have been identified as one the most conserved positions of a fungal LysM consensus pattern (Akcapinar *et al*., 2014). However, only two conserved cysteines at amino acid positions 12 and 43 were identified in one or more of the LysM domains of RcLysM1, RcLysM4, RcLysM6 and RcChi (Figure 4.2). Furthermore, no conserved cysteine pattern was identified in Ecp6 LysM domains. In addition to the conserved cysteines, a WNP motif was identified in the

consensus. The WNP motif (Figure 4.2) was also identified but again in only RcLysM1, RcLysM4, RcLysM6. The Asn (N) amino acid located within the motif is highly conserved and also across the plant and bacterial kingdoms and was found in all LysM domains except for RcLysM6_1.

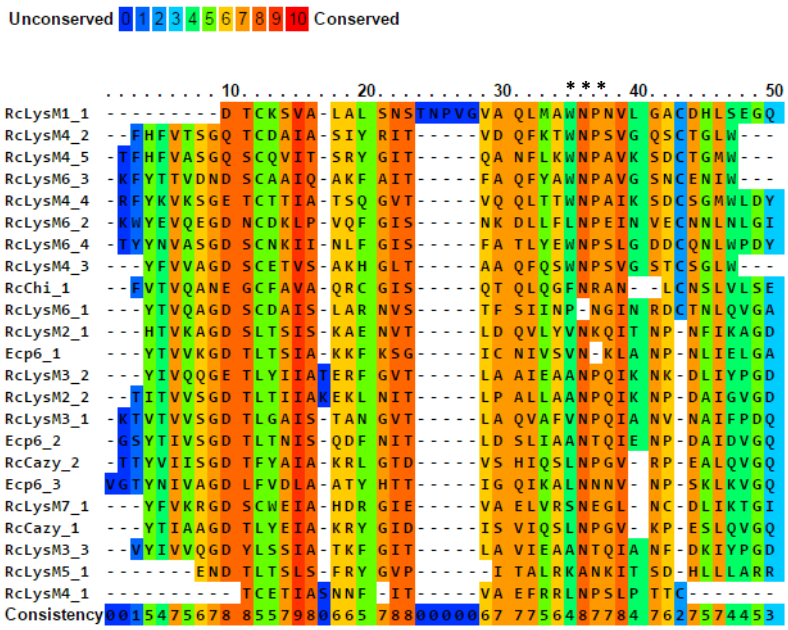


Figure 4.2 PRALINE multiple alignment of all LysM domains identified within *Rhynchosporium commune* proteins against the LysM domains from *Cladosporium fulvum* effector Ecp6 showing conserved amino acids highlighted in orange and red. Asterix indicate the WNP motif position.

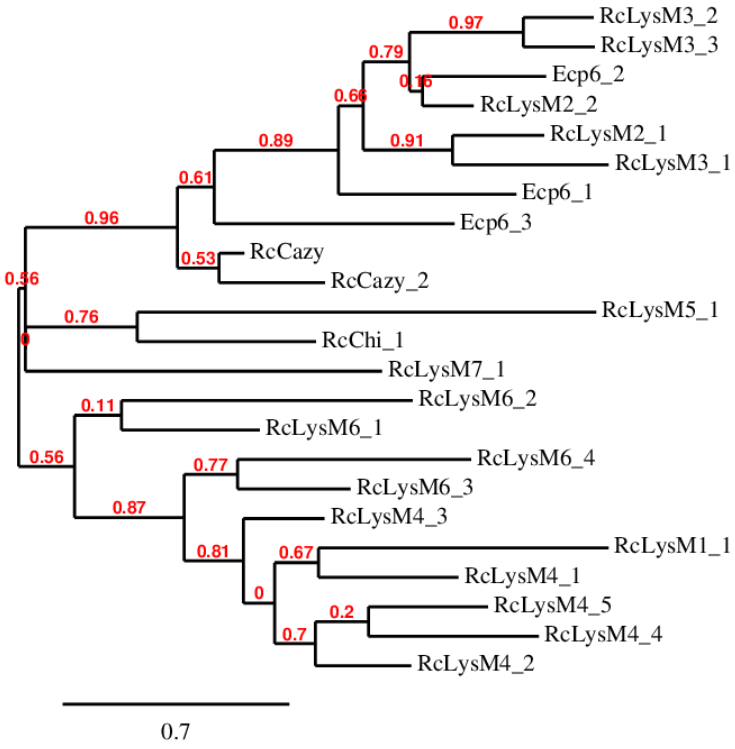


Figure 4.3 Phylogenetic tree showing the relationship between the LysM domains from *Rhynchosporium commune* LysM domain proteins and *Cladosporium fulvum* Ecp6. LysM domains from RcLysM1, 2 and 3 grouped together with the three Ecp6 LysM domains. Bootstrap support values from 1000 replicates are shown at the nodes. The scale bar represents 70 % weighted sequence divergence.

4.2.3 Expression analysis of genes coding for LysM domain proteins reveals upregulation during infection of barley

There is a diverse range of functions for LysM domain proteins in fungi which may not be involved in pathogenesis (Kombrink & Thomma, 2013; Santhanam *et al*., 2013). To determine the function of proteins as potential effectors the expression of *R. commune* LysM domain genes was analysed during infection of barley. Using the same cDNA samples, used in the previous chapter, - from susceptible barley cultivar Optic inoculated with strain L2A or 214-GFP, expression profiles were obtained using the comparative quantification algorithm - ΔΔCt method. *R. commune* actin was used as constitutively expressed endogenous control gene and relative expression of all the genes was normalized against expression levels in conidia (assigned a relative expression value of 1.0).

The expression pattern of the genes in strain L2A were generally similar to those in strain 214-GFP. However, the level of transcript abundance or upregulation was up to 100 times less in 214-GFP and in some instances genes were expressed a day later or earlier (Figure 4.4-4.5). This is expected from different strains as the level of aggressiveness and rate of growth can vary. In addition experiments were not run in parallel which may account for the variation within the levels of expression. *RcLysM4* and *RcCAZy* were either not expressed or expressed at very low levels in all the samples and therefore are not included in the figures. While *RcLysM5* transcript expression was upregulated over 100 times in germinated conidia and over 200 times at 3 dpi in strain L2A to the levels of 10-20 times higher than actin (Figure 4.4), it was not detected in 214-GFP, suggesting that it is either not expressed or the level of transcript abundance was too low to detect. This suggests that *RcLysM5* may not be an essential gene.

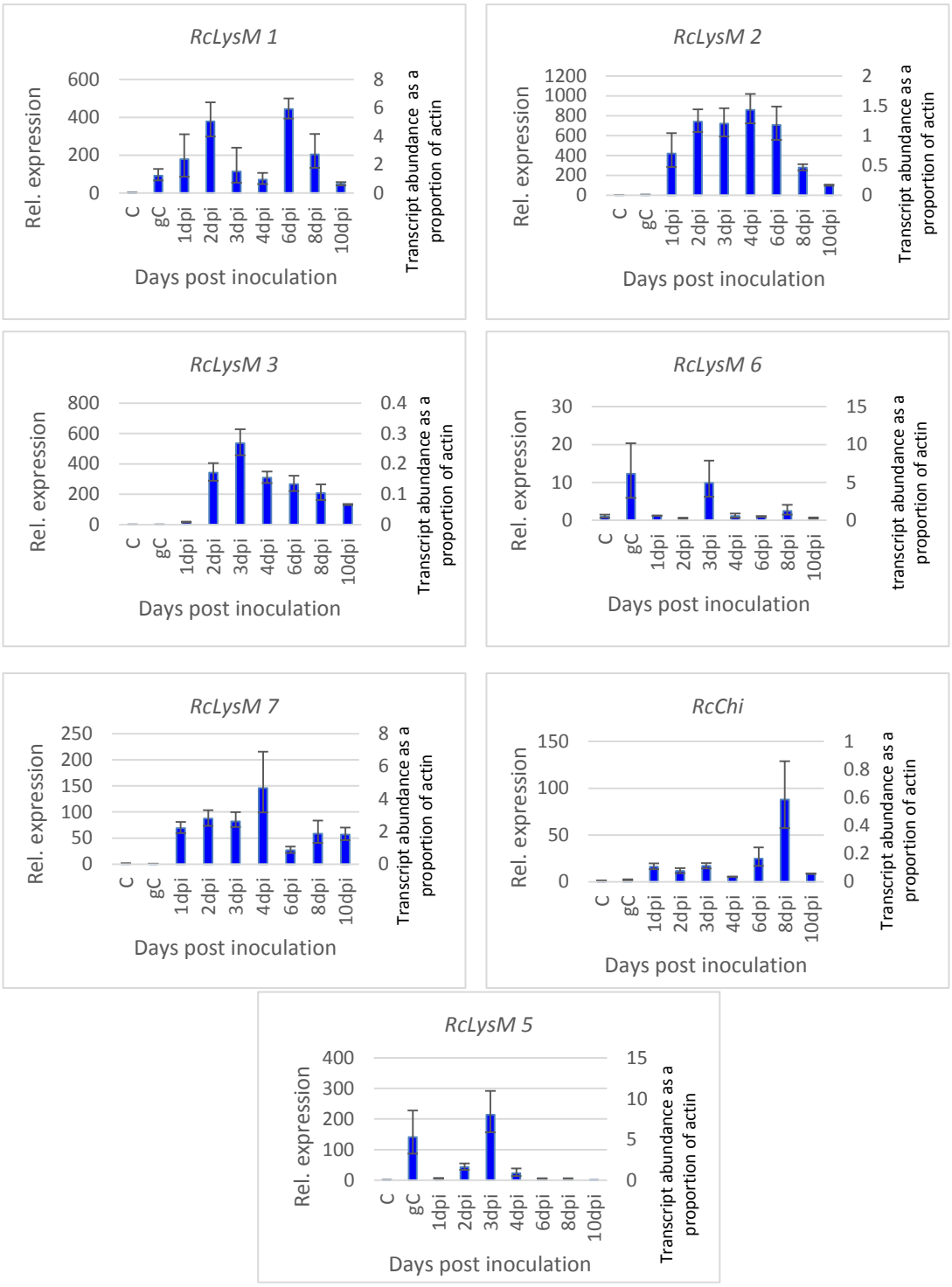


Figure 4.4: Relative expression of genes coding for LysM domain proteins during infection of susceptible barley cultivar Optic with *Rhynchosporium commune* strain L2A. Error bars indicate the confidence intervals for the average of three technical repetitions. All gene expression was normalised against expression in conidia using *R. commune* actin as the endogenous control.

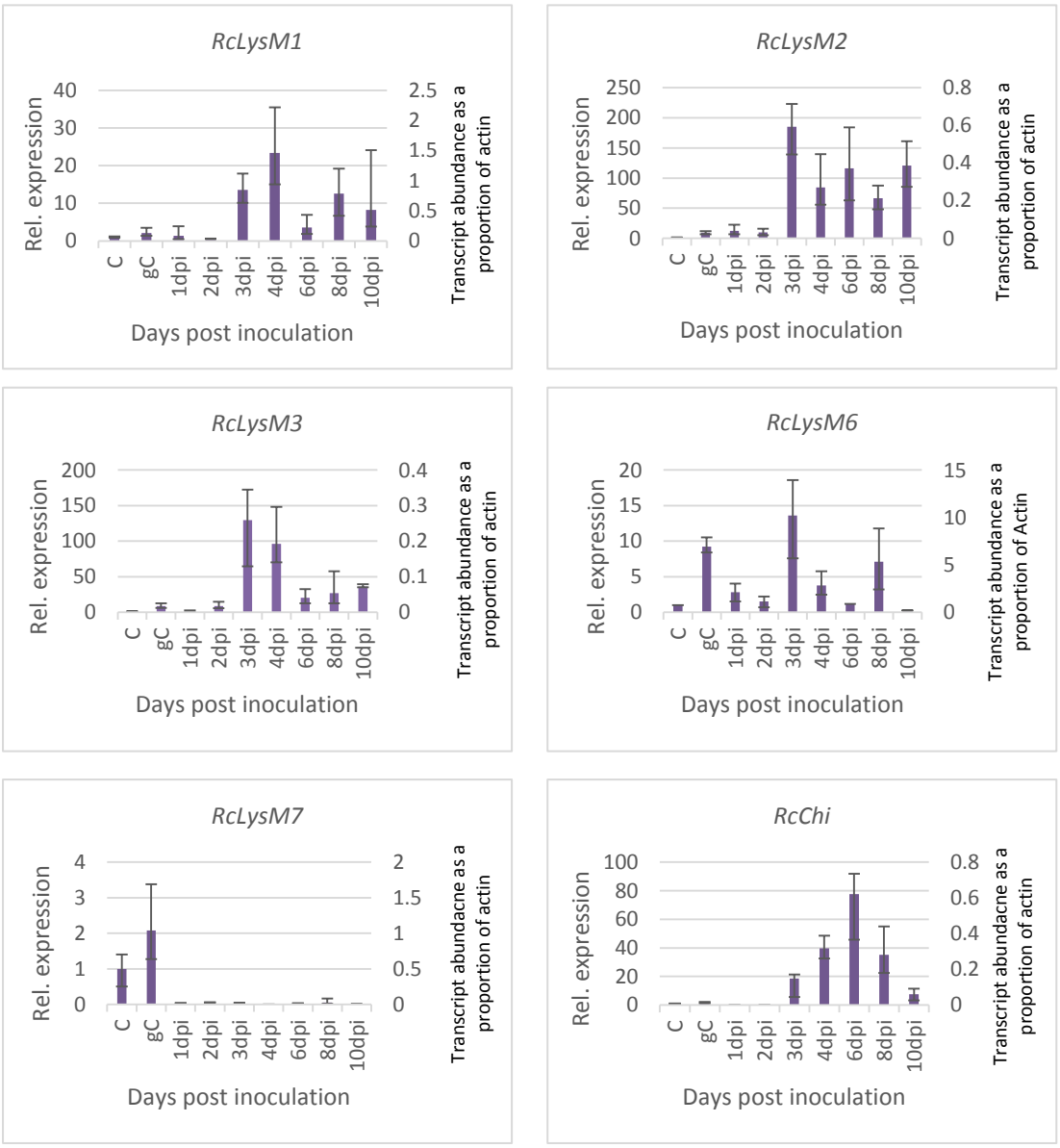


Figure 4.5: Relative expression of genes coding for LysM domain proteins during infection of susceptible barley cultivar Optic with *Rhynchosporium commune* strain 214-GFP. Error bars indicate the confidence interval for the average of two biological repetitions. All gene expression was normalised against expression in conidia using *R. commune* actin as the endogenous control.

Similar to *ChEIp1* and *ChEIp2*, *Mg3LysM* and *Slp1*, a number of genes were highly abundant during the early biotrophic phase (Takahara *et al*., 2016; Mentlak *et al*. 2012; Marshall *et al*., 2011). *RcLysM2* showed an increase in expression from 1 to 6 dpi of 400-800 times compared to its level in conidia to the level similar to that of actin before dropping down from 8 dpi during infection of barley cultivar Optic with strain L2A (Figure 4.4). *RcLysM7* was upregulated 50-150 times throughout first 10 days of the infection with strain L2A to 2-4 times the level of actin (Figure 4.4), and in strain 214-GFP *RcLysM7* transcript was already about as abundant as actin in conidia and germinated conidia, and was downregulated during infection (Figure 4.5).

During infection of barley cultivar Optic with strain 214-GFP *RcLysM2* upregulation started at 3 dpi and continued to at least 10 dpi (Figure 4.5). In contrast, RcLysM3 although expressed throughout the infection had distinct maximum transcript abundance at 3 dpi and 3-4 dpi in strains L2A and 214-GFP respectively (Figure 4.4-4.5). *RcLysM1* upregulation was peaking at two different time points 2 dpi and 6 dpi in strain L2A, and 3-4 dpi and 8-10 dpi in strain 214-GFP (Figure 4.4-4.5).

RcLysM6 was upregulated around 10 times in germinating conidia and at 3 dpi to 5-10 times the level of actin in both strains (Figure 4.4-4.5). In addition to that in strain 214-GFP it also reached the above mentioned level at 8 dpi (Figure 4.5). Only one gene - *RcChi* was found to be highly abundant at the later biotrophic phase, 8 dpi in strain L2A and 4-8 dpi in strain 214-GFP to around 0.5 times the level of actin (Figure 4.4-4.6). However, both *Mg1LysM* and *Ecp6* showed a higher level of expression at 9 dpi and 13 dpi respectively.

All LysM fungal effectors are expressed at a time corresponding to the potential release of chitin fragments from the fungal cell walls into the apoplast and thus may play a role in chitin sequestration. In addition, expression at this stage of infection suggests other possible roles in the colonisation of the plant apoplast aiding in the protection against plant immunity like that of Avr4 (van den Burg *et al*., 2006).

RcLysM1, *RcLysM3* and *RcLysM5* were not upregulated at any stage of growth in media in both strains, while *RcLysM6* and *RcLysM7* were upregulated at 8 dpi in strain 214-GFP, but not in L2A (Figure 4.7-4.8). In contrast, transcript upregulation of *RcLysM2* was evident in both strains (Figure 4.7-4.8). *RcChi* was highly abundant from 1-3 dpi in L2A but peaking much later at 8 dpi in 214-GFP (Figure 4.7-4.8). The presence of transcripts in media suggests a role in growth and morphogenesis. However, it does not unequivocally determine that they are not involved at any stage during pathogenesis as it is becoming clear that LysM effectors can play different roles and can be effective at basal levels during infection (Takahara *et al*., 2016).

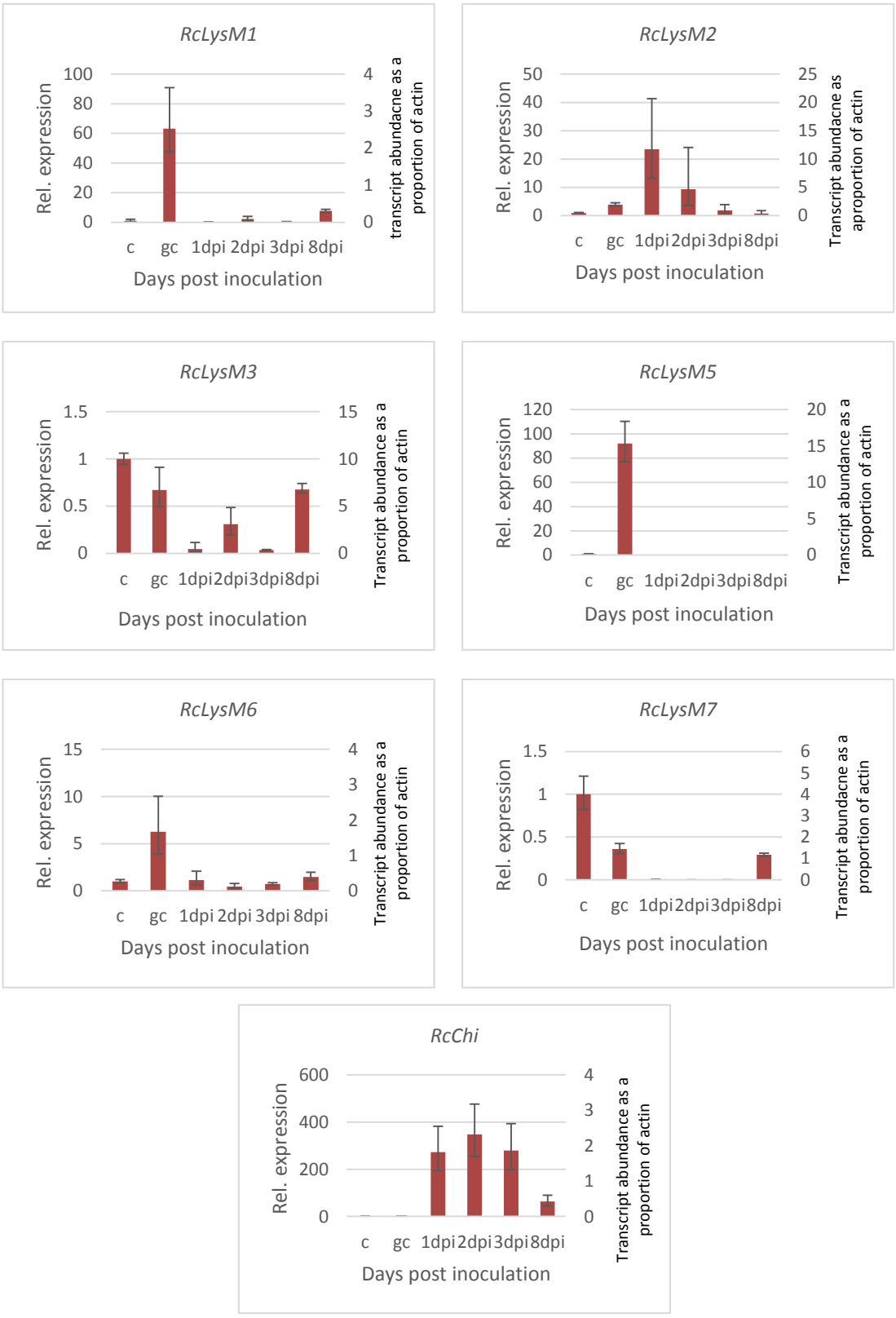


Figure 4.6: Relative expression of LysM genes in *Rhynchosporium commune* strain L2A during its growth in PDB medium. Error bars indicate the confidence intervals for the average of 2 biological repetitions. All gene expression was normalised against expression in conidia using *R. commune* actin as the endogenous control.

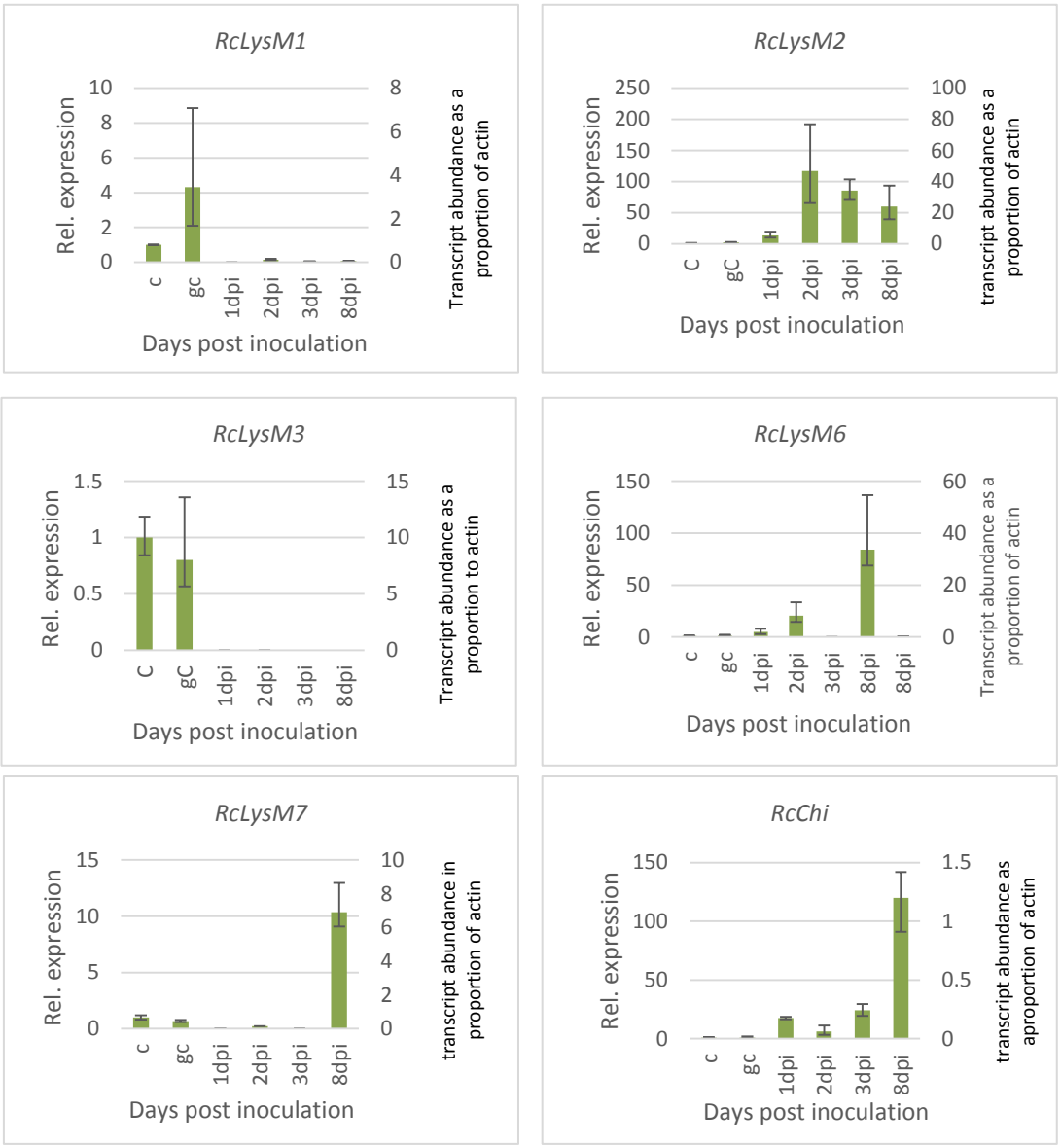


Figure 4.7: Relative expression of LysM genes in *Rhynchosporium commune* strain 214-GFP during its growth in PDB media. Error bars indicate the confidence intervals for the average of 2 biological repetitions. All gene expression was normalised against expression in conidia using *R. commune* actin as the endogenous control.

4.2.4 RcLysM3 protein shares sequence homology with *Z. tritici* Mg3LysM and *C. fulvum* Ecp6

As RcLysM3 contains three LysM domains the same number as CfEcp6 and MgLysM3 which have been both functionally characterised, this protein was selected for further analysis. Conservation could be noted in all three LysM domains between the proteins (Fig.4.8). However, MgLysM3 appeared to share more homology with Ecp6 than RcLysM3 even within the amino acids outside the LysM domains. In addition, an area of around 20 amino acids following the signal peptide and prior to the start of the first LysM domain was not present in RcLysM3. At the same time RcLysM3 has 3 amino acids deletion and 1 amino acid insertion in the second LysM domain, 5 amino acids insertion in the middle of the third LysM domain and an extra 16 amino acids at the C terminus of the protein compared to CfEcp6 and MgLysM3 (Fig.4.8).

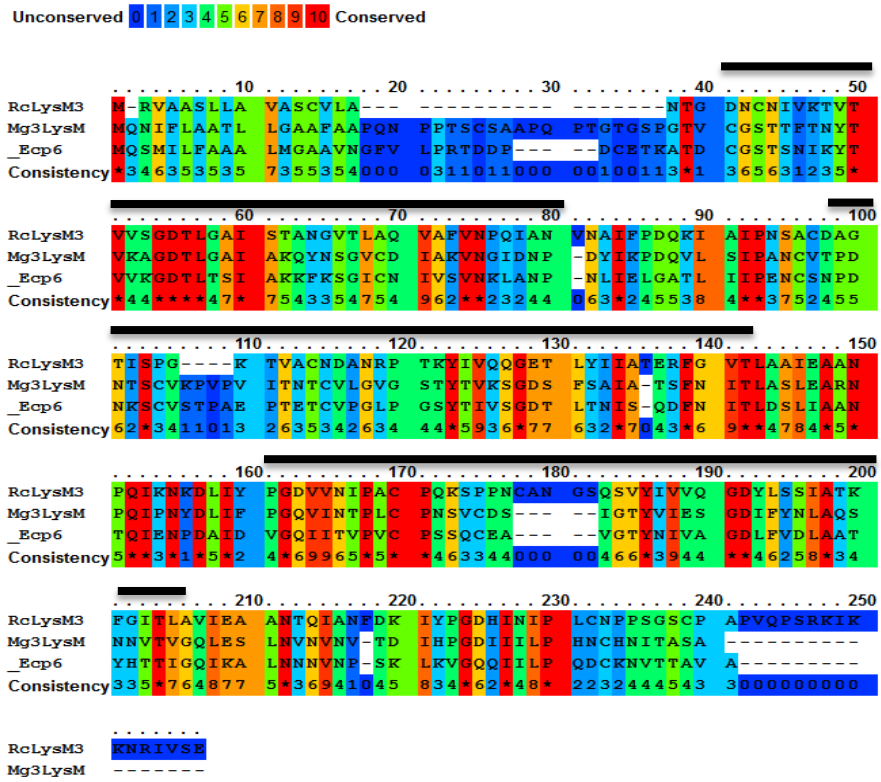


Figure 4.8 Alignment of full length protein sequences of RcLysM3, Mg3LysM and CfEcp6. Highly conserved amino acids are highlighted in red and orange whereas non conserved amino acids are in blue. Black lines indicate the position of the LysM domains

4.2.5 *In vitro* analysis confirms the ability of RcLysM3 to bind chitin

As the crystal structure of *C. fulvum* effector Ecp6 had already been solved, it was possible to model RcLysM3 structure based on that of Ecp6 (Sánchez-Vallet *et al.*, 2013). The presence of three LysM domains in RcLysM3 suggested that it has glycan-binding activity, similar to Ecp6. To give a first insight into the binding capabilities of the RcLysM3 an in silico binding assay was conducted using a prediction based model algorithm (https://www.cgl.ucsf.edu/chimera/) to determine the binding capabilities to the polysaccharide chitin. Twenty-eight ionic bonds, which play an important role in determining the shape of tertiary structures of proteins were predicted. Three hydrogen bonds, which are involved in both the

intra and intermolecular interactions of proteins were also identified. Based on the structural components of RcLysM3 protein, a direct binding groove between the 1st and 3rd LysM domains of RcLysM3 was revealed. A high confidence prediction suggested that chitin oligomer was likely to bind in this groove with binding similar to Ecp6. (Epihov, unpublished data) (Fig. 4.9).

To confirm the chitin binding prediction RcLysM3 protein tagged with V5 peptide at the C terminus to allow detection, was produced in *P. pastoris* and affinity binding to a range of polysaccharides was examined. RcLysM3 co-precipitated with crab shell chitin and, interestingly, with chitosan but not with any of the plant cell wall polysaccharides, xylan or cellulose (Figure 4.10). Almost all LysM effectors identified to date have been shown only to bind chitin. However, Tal6 LysM protein from the soil fungus *Trichoderma viride* also binds chitosan but is involved in self-signalling processes during fungal growth rather than fungal-plant interactions (Seidl-Seiboth, 2013).

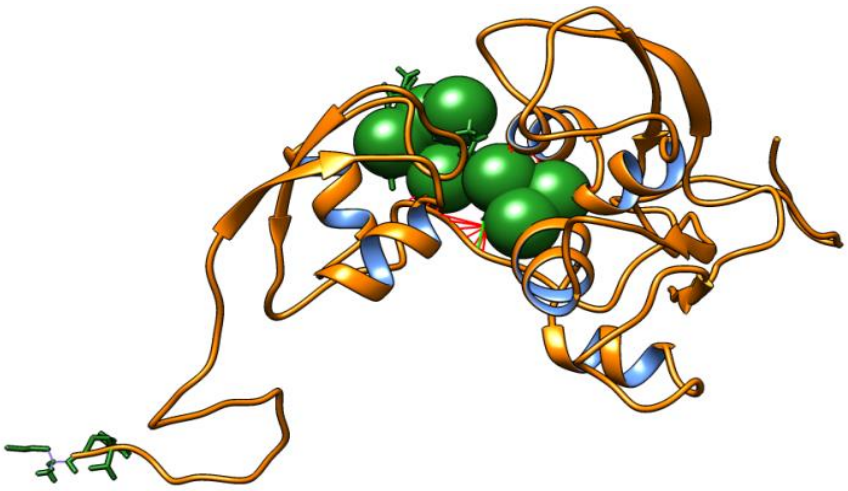


Figure 4.9: Predicted 3D image produced by CHIMERA of RcLysM3 showing three LysM domains containing βααβ tertiary structure associated with LysM domains in orange and the binding groove between LysM1 and LysM3 binding chitin heptamer – green.

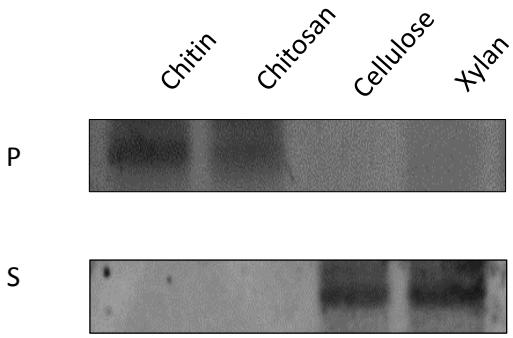


Figure 4.10 Ruby stained protein gel showing RcLysM3-V5 protein co-precipitating in the pellet (P) of chitin and chitosan, but only present in the supernatant (S) of cellulose and xylan.

4.2.6 RcLysM3 does not provide protection to *Trichoderma viride* spores against plant hydrolytic enzymes

It has been previously demonstrated that the *C. fulvum* effector Avr4 contains an invertebrate CBD. Unlike Ecp6, it has the ability to protect against plant hydrolytic enzymes *in vitro* (van den Burg *et al*., 2006; de Jonge *et al*., 2010). To test if RcLysM3 shared the same characteristics to AVR4, the protein was analysed for the protection of *T. viride* spores against plant chitinases. *T. viride* has been shown to be highly susceptible to plant chitinases and has been successfully used in previous experiments (van den Burg *et al*., 2006; de Jonge *et al*., 2010). In addition, *R. commune* grows at a much slower rate and thus the experiment would have proved more difficult. Spores were incubated with RcLysM3 protein and then treated with crude extract of barley leaves containing chitinases. Growth of *T. viride* was clearly inhibited by the hydrolytic enzymes present in this extract. The addition of CfAvr4, but not CfEcp6, was able to protect the fungus against hydrolysis (results not shown). However, RcLysM3 did not share the same function.

4.2.7 Correlation between virulence/avirulence of *R. commune* strains on barley cultivar La Mesita and a SNP change in the RcLysM3 allele.

To identify potential *Avr* genes , predicted *R. commune* effector gene sequences were screened for the presence/absence and/or single nucleotide polymorphisms (SNPs) in genome sequences of 9 *R. commune* strains with different race specificities (Avrova, unpublished data) A SNP leading to a change in an amino acid at position 67 from a Glutamic acid (Q) to Glutamine (E) within the protein sequence of RcLysM3 was identified that correlated with a change in virulence/avirulence of 9 sequenced *R. commune* strains on cultivar La Mesita (Table 4.2). This cultivar alongside other differential lines have been used at the James Hutton Institute to determine the virulence of *R. commune* isolates. (Lynott, unpublished data). Isolate L43D carrying the E allele was avirulent on cultivar La Mesita. A detached leaf assay confirmed the lack of macroscopic symptoms. RcLysM3 sequence was analysed in a further four isolates L101B, L90A, L43A and L43B. Both L101B and L90A contained the SNP resulting in Q allele whereas L43A and L43B contained the SNP resulting in E allele (Table 4.2). The latter two were isolated from the same plant and are possibly the same strain as L43D. While both L101B and L90A isolates containing the Q allele were virulent on La Mesita in line with Q allele being a virulent allele, virulence testing of the isolates L43A and L43B contained the E allele on La Mesita still needs to be conducted to determine if the correlation is valid for these isolates.

Table 4.2: Correlation of the Gln (E) and Glu (Q) allele with the virulence and avirulence of *Rhynchosporium commune* isolates on barley cultivar La Mesita

	R. commune isolates												
	13-13	214	L2 A	L32 B	L43 D	L73 A	L77	UK 7	AU 2	101 B	90 B	L43 A	L43 B
La Mesita	V	V	V	V	A	V	V	V	V	V	V	?	?
RcLysM 3 allele	Q	Q	Q	Q	E	Q	Q	Q	Q	Q	Q	E	E

Prior to the end of the project, plasmids were created for the over expression of both alleles to transform L43D with the potentially virulent Q allele and AU2 highly virulent strain - with the potentially avirulent E allele, which will help to determine if the SNP has an effect on structure and/ or recognition.

Discussion

It is clear from the literature that genes coding for LysM effector proteins are a ubiquitous feature of fungal genomes and LysM effector proteins play an invaluable role in the survival of fungal pathogens in the apoplast. A large number of proteins with LysM motifs are continually being identified as more fungal genomes are being unravelled. *R. commune* genome contains an expanded family of genes coding for a range of LysM domain proteins from RcLysM1, RcLysM5 and RcLysM7, containing a single LysM domain, to RcLysM2 and RcLysM3, containing 2 and 3 LysM domain respectively, which are the most common types of LysM domain proteins identified in other plant pathogens (Bolton *et a*l., 2008), RcLysM4 and RcLysM6 containing 5 and 4 LysM domain respectively, as well as a putative chitinase containing LysM and a chitin-binding domain and a protein with 2 LysM and a lysozyme-like domain.

Although the functional range of these proteins is only partially discovered so far, it has been shown that many fungal pathogens use different LysM proteins for various functions during pathogenesis (van der Burg *et al*., 2006; de Jonge *et al*., 2010; Marshall *et al*., 2011). Through the utilisation of sequence prediction tools and *in planta* expression analysis, it is possible to suggest that some of *R. commune* LysM domain containing proteins may be involved in pathogenesis.

Expressed in germinated conidia and at 3 dpi in planta, RcLysM5 and RcLysM6 may have roles during germ tube formation and possibly penetration of the cuticle probably in protecting the emerging germ tube. This function has recently been demonstrated for the two LysM proteins from the ascomycete fungus *C. higginsanium* which are not only essential for appressorium-mediated penetration

but for fungal virulence as well (Takahara et al., 2016). At a much later time point during the biotrophic phase, RcChi was highly abundant. A chitinase domain and a CBD identified in this protein, coupled with the late upregulation may indicate a role in hyphal growth and remodelling (Langer *et al.*, 2015). The protein may be involved in perturbing the recognition of chitin by plants during hyphal growth. This could be explained through the presence of both domains – CBD, that binds chitin fragments released from the fungal cell wall, and the chitinase domain functioning to break down the chitin into smaller fragments which would not be recognised by plant PRRs.

Most LysM effectors have been shown to be upregulated during the infection of the apoplast and this was the case for *RcLysM1*, *RcLysM2*, *RcLysM3* and *RcLysM7*. This provides one piece of evidence that can be used to determine if any of the *R. commune* proteins are involved in chitin sequestration during infection of the apoplast. In contrast, not all LysM proteins were highly abundant, this includes RcLysM4 and RcCAZy which were not found to be expressed during *R. commune* growth *in vitro* or *in planta*, indicating that they might be non-functional (in case of RcCAZy), or require other stimuli to induce their transcription.

The LysM motif in fungal species has been shown to contain two different conserved domains – WNP motif and four conserved cysteines (Akcapinar *et al*., 2014) It was interesting to find that some of the *R. commune* proteins didn't contain this protein signature, including RcLysm3. The absence of the protein signature may point towards evolutionary diversity of LysM proteins as fungal effectors as the motifs are also absent in the fungal effectors Ecp6 and Slp1.

Despite the intriguing functions that *R. commune* LysM proteins may possess, it was not possible to characterise all LysM proteins and thus, based on the similarities to CfEcp6, RcLysM3 was selected for further analysis.

In contrast to candidate effectors that share no homology to known effectors from other species, it was possible to use a diverse range of analyses to predict the function of RcLysM3. Initially chitin binding prediction algorithms determined by the threading of RcLysM3 protein onto the CfEcp6 template showed the potential binding capabilities of the protein. Furthermore, alternative methods for functional characterisation were also accessible other than gene knockouts, which from the previous chapter were shown to be inefficient. RcLysM3 was highly expressed *in planta* and through binding analysis revealed that it does in fact bind chitin and therefore it is likely that similar to other effectors, the protein may prevent the plant PRRs recognising the fragments of chitin (Sánchez-Vallet *et al.*, 2014).

To fully determine if the chitin binding function is utilised by the pathogen to outcompete the plant host receptors for chitin binding more comprehensive assays are required. Both Marshall *et al*. (2011) and Mentlak *et al*. (2012) showed that Mg3LsyM and Slp1 outcompeted the host for chitin binding through the suppression of chitin triggered immunity in plant cells. Additional characterisation of CfEcp6 revealed that the crystal structure of this protein had an ultra-high binding affinity and a novel binding strategy, with the dimerization of LysM1 and LysM3 confirming the effector function of the protein (Sánchez-Vallet *et al*., 2013).

While RcLysM3 was shown to bind chitin and chitosan, it failed to protect *T. viride* spores against plant chitinases. This function is shared by several other LysM domain proteins including CfAvr4, Mg1LysM and Mg3LysM but not by CfEcp6

which sequence is much closer to that of RcLysM3 (van den Burg *et al*., 2006; de Jonge *et al*., 2010; Marshall et al., 2011).

It is important to remember that plant pathogens also encounter and interact with a range of microbes at different stages of their lifecycle, including endophytes, commensals and other pathogens (Berg *et al*., 2014). Many of the *R. commune* LysM proteins identified could also provide protection against microbial activity. Hence the expression *in planta* may reflect the roles of the LysM during this time and not just in pathogenesis, providing protection against chitinases produced by mycoparasites. They may also have functions in the general physiological processes such as cell wall modification and growth (Adams, 2004 ;Jonge & Thomma, 2009). This may be a potential role for the *R. commune* LysM proteins expressed in media. LysM proteins with unknown binding affinities may also be able to bind a wide range of polysaccharides, including peptidoglycan, allowing the fungus to challenge any bacterial competitors. This may be likely as the LysM domain was originally identified in bacterial proteins that break down cell walls (Garvey *et al*., 1986; Buist *et al*., 2008).

The persistence of the LysM domain effector proteins in fungal pathogens indicates an essential role for these proteins. In fact, Slp1 and Mg3LysM were shown to be essential for pathogenicity (Mentlak *et al*., 2012; Marshall *et al*., 2011). In light of this it is tempting to speculate that *R. commune* LysM effectors may be good candidates for avirulence gene discovery. Furthermore, the discovery of the correlation between RcLysM3 alleles and virulence/avirulence of different *R. commune* isolates on barley cultivar La Mesita may point towards a potential avirulence gene in *R. commune*. A single amino acid change from Gln to Glu has been shown previously to have an effect on protein function (Clarke *et al*., 1990).

In addition, a study conducted on a peptidase (PepN) from *E. coli* showed that the change from Gln to Glu led to a catalytically inactive PepN (Das *et al*., 2016). The mutation from a Gln to Glu introduces a change in the physical properties of the amino acid. Gln is a neutral uncharged amino acid whereas Glu is acidic and polar charged. Therefore, the mutation results in a loss of charge for the potentially virulent RcLysM3 allele and may cause loss of interactions with other molecular residues (Alanazi *et al*., 2011). This may mean that the E allele is unable to bind chitin as effectively as the Q allele. On the other hand, the change may be associated with the evolution of the effector to prevent recognition from the host R protein (Joosten *et al*., 1997; Na *et al*., 2013; Yin *et al*., 2013). Over expression of alternative alleles of RcLysM3 will help to determine if the SNP has an effect on structure and/ or recognition. Plant recognition of the effectors may result in a longer lasting resistance that could be used in future for the protection of barley against *R. commune*. However, there is already evidence that LysM effectors can evade recognition whilst still retaining function (van Esse *et al*., 2007). It may be possible in the future to incorporate a wide range of *R* genes into barley recognising different LysM alleles resulting in a more robust resistance strategy.

CHAPTER 5

5 Analysis of barley resistance to *R. commune*

5.1 Introduction

Identifying new barley resistance to *R. commune* has become a top priority since the breakdown of *Rrs1* resistance occurred (Schürch *et al*., 2004; Zhan *et al*., 2008). However, due to the pathogens high genetic variability, one of the biggest challenges is finding cultivars with longer lasting resistance (Zaffarano *et al*., 2006). Despite the economic importance of the disease no *R* genes have been cloned and the understanding of a resistant response is limited to the *Rrs1-AvrRrs1* interaction (Rohe *et al*., 1995).

Evaluation of cultivar resistance has generally been scored using qualitative and subjective methods based upon the presence of visual disease symptoms on barley plants after inoculation with the pathogen (Ayliffe *et al*., 2013). However due to long asymptomatic phase of infection this approach fails to provide much insight into asymptomatic infection and how the pathogen is colonising in the response of the host. Molecular diagnostics provides an alternative route for the detection of plant pathogens on asymptomatic hosts and has been successful in identifying *R. commune* in symptomless seed (Lee *et al*., 2002). Furthermore, quantitative molecular techniques to measure biomass accumulation in infected plant leaves have also been useful. Fountaine *et al*., (2007) showed correlation between the lowest levels of *R. commune* biomass and the cultivar with the lowest resistance

rating. However, this was not the case for the levels of disease in all cultivars tested.

To provide a better understanding of resistance to *R. commune* the response of the GFP transformed isolate 214 (carrying the NIP1 gene) to susceptible cultivar Atlas and resistant cultivar Atlas 46 containing the *Rrs1* gene were compared (Thirgnanasbanadam *et al*., 2011). During an incompatible interaction the pathogen was shown to be highly restricted in growth and a change in fungal morphogenesis characterised the *Rrs1* resistance response.

Further research of the *Rrs1* resistance mechanism identified proteins deleterious to the cell wall of *R. commune* conidia from resistant cultivar Atlas 46 infected with a strain of *R. commune* (Zareie *et al*., 2002). However, there is no information in relation to the presence of the *AvrRrs1* gene in this *R. commune* strain and no evidence of the infection process. Another study highlighted the induction of pathogenesis related (PR) genes during an incompatible interaction with Atlas 46 and in response to the Avr protein NIP1 compared to the near isogenic (NIL) line Atlas (rrs1). PR-1, PR-5, and PR-9 were shown to be specifically upregulated earlier and to a higher level in the mesophyll of resistant plants whereas PR-10, LoxA and pI2-4 were specifically induced in the epidermis of resistant plants (Steiner-Lange *et al*., 2003).

The establishment of defence requires the fine regulation of a wide variety of apoplastic proteins which can act rapidly and effectively to restrict pathogen's spread. Some studies have used proteomics to screen the apoplast for proteins

involved in resistance, identifying extracellular enzymes involved in defence and cell wall metabolism (van der Westhuizen *et al*., 1998; Floerl *et al*., 2008; Delaunois *et al*., 2012)

It has become evident that numerous approaches are required to obtain a more detailed picture of resistance and to gain a better understanding of the type of resistance barley confers against this pathogen. Furthermore, our knowledge is still limited regarding the mechanisms of other barley major *R* gene resistance to this pathogen. To investigate the mechanisms of other barley resistant genotypes this research takes advantage of using fluorescent confocal microscopy, to visualise growth of *R. commune* during infection on barley lines containing *R* genes other than *Rrs1*. In addition, a comparative proteomic approach to identifying proteins present during an *Rrs1* resistant interaction may highlight some interesting proteins that can be used to assess the resistance of other barley genotypes.

5.2 Results

5.2.1 *Rrs3, Rrs4* and *Rrs13* show a high level of resistance *to R. commune* isolates

Detached barley leaves were inoculated with a conidial suspension of *R. commune* to obtain a phenotype of each barley line. A total of nine sequenced strains were used to infect a set of barley lines containing the resistance genes *Rrs3*, *Rrs4* and *Rrs13.* Each assay included a very susceptible cultivar Optic as a control to determine isolates' pathogenic capabilities. Inspection of lesion formation began around 10 dpi and lesion measurements continued until 21dpi.

Results showed that all barley lines were susceptible to L77 and AU2 which were the most virulent in comparison to other strains. Strain AU2 caused early lesions development and was virulent on barley lines Abyssinian (*Rrs3*), CI11549 (*Rrs4)* and BC Line30 (*Rrs13).* Abyssinian, CL11549 and BC line 30 were also susceptible to strain L77. Susceptible barley lines inoculated with strain L77 also developed lesions quickly and produced symptoms that were comparable to the highly susceptible control Optic, although lesions did take longer to develop on Cl11549 which contains the *Rrs4* gene. In contrast strains UK7, L32B, L43D, L73A and 214-GFPcaused no lesions on barley plants containing *Rrs3* or *Rrs4* and *Rrs13.* The lack of lesions may indicate the presence of resistance which can be further assessed. Results from all detached leaf assays are detailed in table 5.1.

Table 5.1: Virulence testing results of barley lines containing *Rrs3 (*Abyssinian), *Rrs4* (Cl11549) & *Rrs13* (BC Line 30) resistance genes inoculated with *R. commune* strains L32B, L43D, L73A, L77, UK7, AU2 & 214-GFP, at 21 days post inoculation. A = avirulent – no lesion was present on the barley leaf inoculated with the *Rhynchosporium commune* isolate throughout the experiment. V -= virulent – lesions were present by the end of the experiment on the barely leaf inoculated with the *Rhynchosporium commune* isolate. The susceptible barley cultivar Optic was used as a control. Experiments were conducted between three and five times.

Barley *R* gene	R. commune isolates						
	L32B	L43D	L73A	L77	UK7	AU2	214-GFP
Optic							
	V	V	V	V	V	V	V
Abyssinian *Rrs3*							
	A	A	A	V	A	V	A
Cl11459 *Rrs4*							
	A	A	A	V	A	V	A
BcLine 30 *Rrs13*							
	A	A	A	V	A	V	A

Experiments conducted with strains 13-13, 214 and L2A did not result in the formation of lesions and there were no lesions produced on the susceptible control throughout the assay indicating the results were not valid. The isolates were tested at higher inoculation levels but showed no difference in pathogenicity. Strains 13-13, 214 and L2A were tested on a three separate occasions but due to the probable loss of pathogenicity due to the prolonged cultivation on media, it was not possible to continue using these isolates. Strain 214-GFP produced no visible lesions throughout the assay on all three barley lines BcLine 30, Cl11549 & Abyssinian.

5.2.2 Analysis of asymptomatic infection on barley lines using *R. commune* strain 214-GFP

Barley lines containing *Rrs4* and *Rrs13* showed a moderately high level of resistance in terms of lack of lesion formation to 5 out of 7 *R. commune* strains. Further analysis to determine how the fungus proliferates during asymptomatic infection was conducted using *R. commune* strain 214-GFP.

Microscopic analysis began at 2 dpi to determine if plant resistance was affecting germination which has been previously described as a mechanism of resistance (Lehnackers & Knogge, 1990). At 2 dpi the conidia appeared normal and germination was visible on all barley backgrounds, suggesting little or no effect on conidial germination (Figure 5.1 A, C, E).

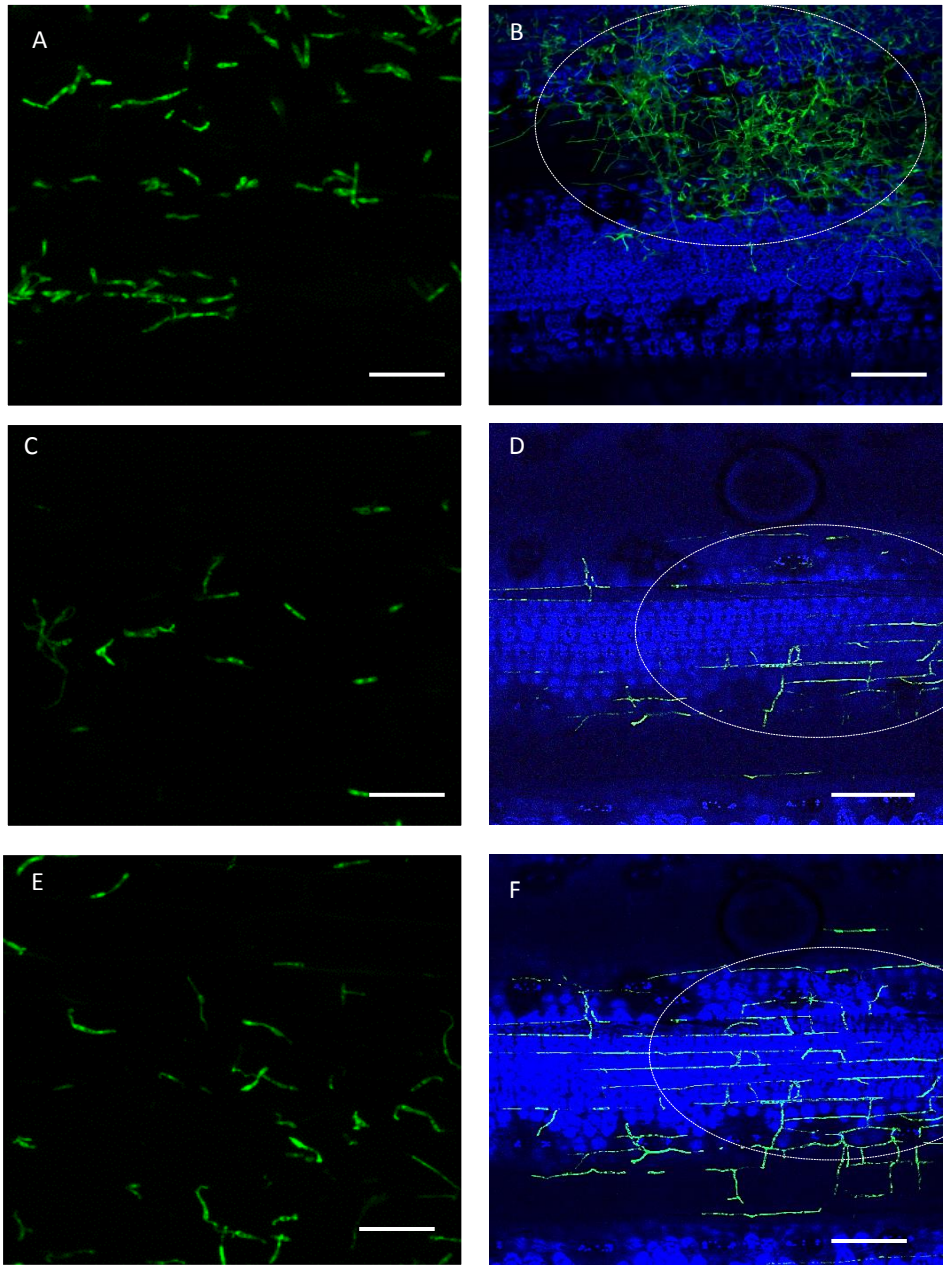


Figure 5.1 Confocal LASER microscopy images of the infection progress following inoculation with *Rhynchosporium commune* strain 214-GFP infection. A – *R. commune* spores & B- growth within the inoculum spot on barley line CI11549 (*Rrs* 4); C- *R. commune* spore & D- less growth then the susceptible Optic in F with some growth out with the inoculum spot on barley on BC Line 30 (*Rrs* 13) and E- *R. commune* spores & F- colonisation of the epidermal tissue of the susceptible barley cultivar Optic. GFP excitation of 488 nm and emission collection of 500-530 nm. The autofluorescence signal from plant chlorophyll was collected with an emission range of 650-700 nm. Images are representative of 5 experimental repetitions. Scale bars A, C, E = 50µm & B, D, F = 100 µm. White circles indicate the inoculum spot.

Growth after 10 dpi was investigated to determine the extent of the mycelial network. In comparison to the susceptible barley line the amount of growth at 10 dpi was much less for BC Line30 carrying the *Rrs13* resistance gene (Figure 5.1 D). Although the growth was less, it followed the same pattern of growth as seen in a susceptible cultivar (Figure 5.1 F). Despite that pathogen growth on *Rrs4* line Cl11549 was evident, the type of growth differed. Instead of the mycelium forming lines between the epidermal cells, the fungal growth was random. The mycelium didn't travel far from the inoculation spot suggesting line Cl11549 to be resistant to strain 214 (Figure 5.1 B). The inoculum spot is highlighted in Figure 5.1 by a white circle.

5.2.3 Analysis of asymptomatic infection of barley landraces using *R. commune* strain 214-GFP

In addition, a further two barley lines were analysed for asymptomatic growth. Syrian landraces were used to look at response to infection and were included in this research as they are genetically more diverse than cultivated barley which increases the chance of finding novel barley resistance (Ceccarelli *et al*., 1987).

It was evident that the interaction between SLB 66_024 (Unknown *R* gene) and 214-GFP was not compatible. The early stages of growth showed a similar pattern to a resistant line (Figure 5.2 A) and although there was quite a substantial amount of growth at 21 dpi the mycelium did not grow along the epidermal cell walls (Figure 5.2 C). Instead, the growth was randomly dispersed. In contrast, growth of 214-GFP on SLB67-015 (Unknown *R* gene) was established after 8 dpi and continued throughout the assay resulting in a bidirectional direction of mycelium growth out with the inoculum spot by 21dpi (Figure 5.2 D). The pattern of growth was similar to a susceptible interaction (Figure 5.2 B).

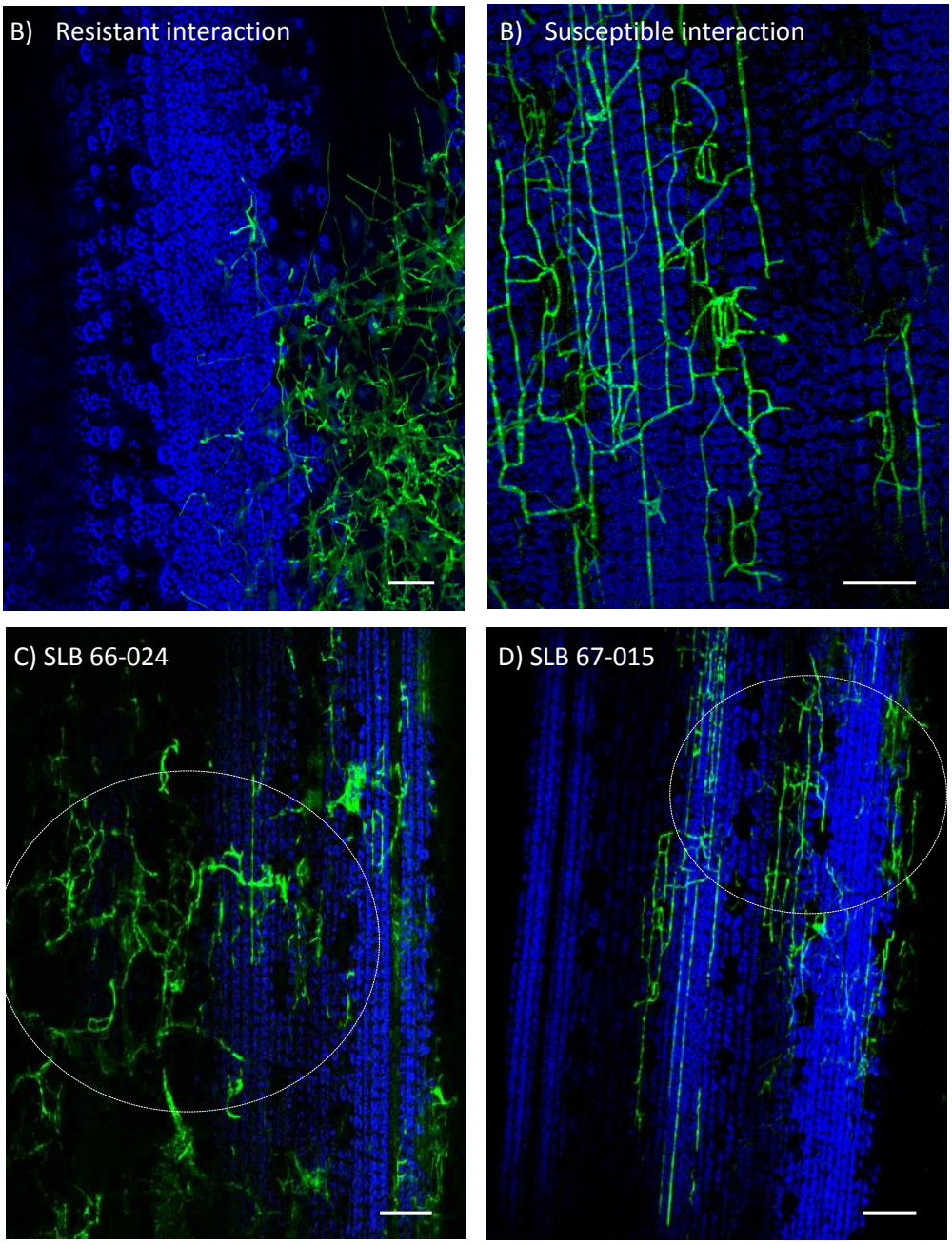


Figure 5.2 Confocal LASER microscopy images of *Rhynchosporium commune* strain 214-GFP infection on C) SLB 66-024 and D) SLB 67-015 D at 21dpi. The growth of *R. commune* on SLB 66-024 was similar to a resistant interaction shown in A (Atlas46 resistance to isolate 214 GFP). Growth on barley line SLB 67-015 was similar to a compatible interaction shown in B (Optic susceptibility to isolate 214 GFP), where colonisation occurs around the epidermal cells. White circles represent the inoculum spot. GFP excitation of 488 nm and emission collection of 500-530 nm. The autofluorescence signal from plant chlorophyll was collected with an emission range of 650-700 nm. Images are representative of 5 experimental repetitions. Scale bars A, B, C &D= 100µm

5.2.4 Quantitative proteomics

Although microscopy can distinguish between lack of growth and the presence of morphological differences, to gain a better understanding of the molecules involved in resistance to *R. commune*, a quantitative proteomics approach was taken to determine the change in abundance or absence of proteins.

Three biological experiments were used for the extraction of infected and uninfected apoplast extract. The infection of inoculated cultivars was analysed using *R. commune* strain 214-GFP. To confirm colonisation of the leaves of susceptible cultivar Optic, partially resistant Atlas and restricted growth on the leaves of resistant cultivar Atlas 46 leaf samples were viewed under confocal microscope before taking samples for apoplastic extraction for quantitative proteomes. Growth of 214-GFP was as expected (Thirgnanasbanadam *et al*., 2011) – Optic contained the highest level of colonisation (Figure 5.3 A & B) whereas resistant Atlas 46 showed very restricted growth with random colony morphology (Figure 5.3 E&F), growth was identified on Atlas (Figure 5.3 C & D) but not to the extent of Optic.

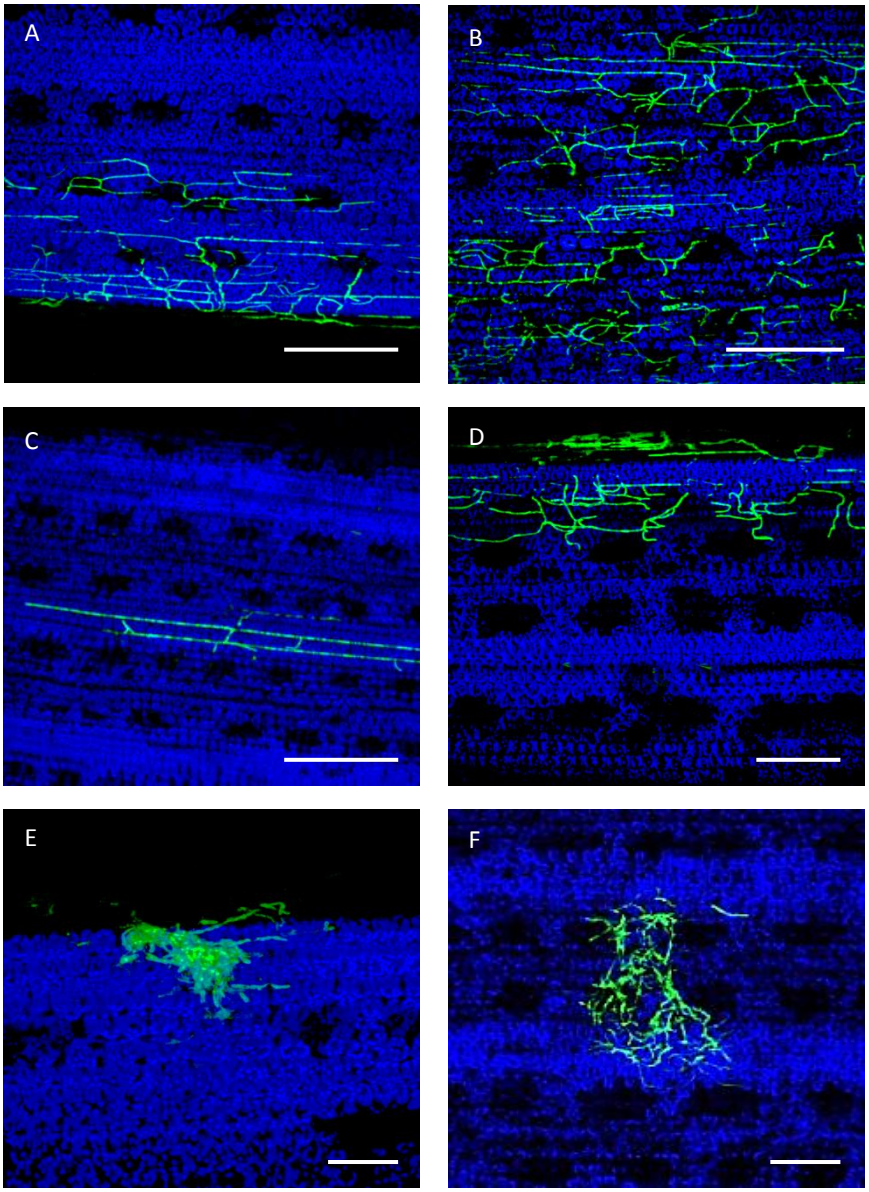


Figure 5.3: Confocal LASER microscopy images of *Rhynchosporium commune* strain 214-GFP infection at 4 dpi and 7 dpi on susceptible Optic (A & B), partially resistant Atlas (C & D) and resistant Atlas 46 (E & F). GFP excitation of 488 nm and emission collection of 500-530 nm. The autofluorescence signal from plant chlorophyll was collected with an emission range of 650-700 nm. Scale bars A, B,C,D = 100µM, E= 50 µM & F=25 µM

Dimethyl labelling was used for quantitative proteomics. Unfortunately, statistical analysis using Perseus revealed that only one of the biological repetitions was suitable to use. The other two repetitions did not follow a normal distribution and therefore were omitted from this work. It was later established that the reason was due to inefficient labelling prior to MS analysis. In addition, the amount of plant proteins identified was extremely low, likely a loss during the labelling procedure. Nevertheless, a number of interesting proteins involved in defence related functions were identified and selected for further analysis.

5.2.4.2 Sequence analysis of defence related proteins identified in barley apoplastic proteome

A total of 49 barley proteins were identified in the infected and non-infected apoplastic fluid of cultivars Optic, Atlas and Atlas46 using the barley genome database (http://pgsb.helmholtz-muenchen.de/plant/barley/). Thirteen of the barley proteins potentially involved in plant defence were selected for further analysis. The remaining proteins are listed in table 9.4.7 in the appendix. The protein sequences were analysed for the presence of a signal peptide (http://www.cbs.dtu.dk/services/SignalP/) and a transmembrane domain (http://www.cbs.dtu.dk/services/TMHMM/). In addition, TargetP (http://www.cbs.dtu.dk/services/TargetP/) was used to predict localisation. BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify similar plant protein sequences. Disease resistance protein and Glucan endo-1,3-beta-glucosidase contained no signal peptide and the predicted target location was unknown which may indicate the proteins maybe secreted via an unconventional secretory pathway. All other proteins were predicated to contain a signal peptide

and were predicted to be secreted via the secretory pathway. However, thaumatin-like protein, chitinase, serine carboxypeptidase and (1-3) beta-glucanase all contained a transmembrane domain. This suggests that the proteins are likely to cell wall anchored proteins and it is possible fragment of the proteins may have been released during apoplastic extraction. Nonetheless, they are active within the apoplast and are important in defence against pathogens.

Table 5.2: Sequence analysis of potential defence related proteins identified in the barley apoplast

Protein Id	Sp	Tm	Tp	Best Blast hit	e-value	Accession #
MLOC_56099.3	28-29	-	S	Arabinoxylan arabinofuranohydrolase isoenzyme AXAH-I [*H. vulgare*]	0	AAK21879.1
MLOC_54205.1	29-30	-	S	PREDICTED: probable Beta-D-xylosidase 7 [*B. distachyon*]	0	XP_003576084.1
MLOC_65311.2	21-22	7-28	S	Chitinase [*H. vulgare* subsp. *vulgare*]	0	ACJ68105.1
MLOC_10425.2	NO	-	-	Disease resistance protein RPM1 [*A. tauschii*]	0	EMT16497.1
AK248896.1	NO	-	-	Glucan endo-1,3-beta-glucosidase GII precursor [*T. aestivum*]	1E-157	CBH32609.1
MLOC_73077.1	24-25	13-35	S	(1-3)-beta-glucanase [*H. vulgare*]	0	EMS57010.1
MLOC_10319.1	26-27	-	S	Purple acid phosphatase 2 [*T. urartu*]	0	EMT05424.1
MLOC_58156.1	24-25	-	S	Subtilisin-like protease [*Aegilops tauschii*]	4.00E-72	EMT11726.1
AK251422.1	22-23	5-24	S	Thaumatin-like protein TLP4 [*H. vulgare*]	0	AAK55323.2
MLOC_75626.1	NO	78-100	S	Serine carboxypeptidase II-3 [*A. tauschii*]	4.00E-72	EMT05424.1

5.2.4.3 Analysis of protein expression reveals upregulation of (1-3) β-glucanase and α-L arabinofuranosidase in Atlas 46 4 dpi

The intensity of each of the proteins was compared in 3 different cultivars, highly susceptible Optic, Atlas 46 which contains the *Rrs1* and *Rrs2* gene and the NIL Atlas which doesn't contain the *Rrs1* gene, uninfected and infected with *R. commune* strain 214-GFPat 4 dpi and 7 dpi.

Four proteins were highly abundant and showed a distinct increase in infected apoplastic samples of Atlas 46 at 4 dpi (Figure 5.4). At 4 dpi the amount of (1-3) beta-glucanase increased 10 fold in Atlas 46 compared to uninfected sample before dropping back to the original level by 7 dpi. At the same time, it increased only 4 fold in Atlas, and remained constant over that period of time in both infected and uninfected Optic (Figure 5.4). This is in agreement with a previous study which identified the gene expression of this protein was higher in a resistant backcross line than in the susceptible parent (Roulin *et al*., 2007). In addition, (1-3) beta-glucanase is activated by SA which induces plant defence against biotrophs, therefore the expression at 4 dpi would confirm this (Glazebrook, 2005). α-L arabinofuranosidase showed a 2-fold increase at 4 dpi in Atlas 46 compared to uninfected sample before dropping back to the original level by 7 dpi. At the same time, it remained constant over that period of time in both infected and uninfected Atlas and Optic (Figure 5.4). Serine carboxypeptidase abundance was around 7 fold higher in Atlas 46 at 4 dpi in comparison to Optic and also present at 7 dpi in uninfected Optic (Figure 5.4). B-glucosidase was only identified in infected apoplast samples at both time points with similar levels in all the cultivars (Figure 5.4). The remaining six proteins were most highly expressed in 7 dpi infected apoplast samples (Figure 5.5). Thaumatin-like protein was not identified at 4 dpi in

uninfected Optic and in any of the infected samples, but showed similar levels in all the other samples (Figure 5.5) The purple acid phosphatase was only detected at 4 dpi. It was much more abundant in Atlas and Optic than in Atlas 46, where it remained at a low level following inoculation. In Optic and Atlas, it increased 3 fold following infection compared to the levels in uninfected leaves (Figure 5.5) This was similar for subtilisin-like protease, which remained at a low level in Atlas 46, but showed a 40-fold increase in Optic and over 60-fold increase in Atlas in infected leaves at 7 dpi (Figure 5.5). In contrast to other studies chitinase was barely expressed in Atlas 46 and again was more abundant in Optic in infected leaves at 7 dpi (Figure 5.5). At 7 dpi the disease resistant protein was upregulated in all cultivars, as expected it was notably higher in Atlas 46 with a fold increase of 1.5 and 2.5 in comparison to Atlas and Optic respectively (Figure 5.5). Glucan endo-1,3-β glucosidase showed similar abundance in apoplastic fluid from all three cultivars at both time points (Figure 5.5).

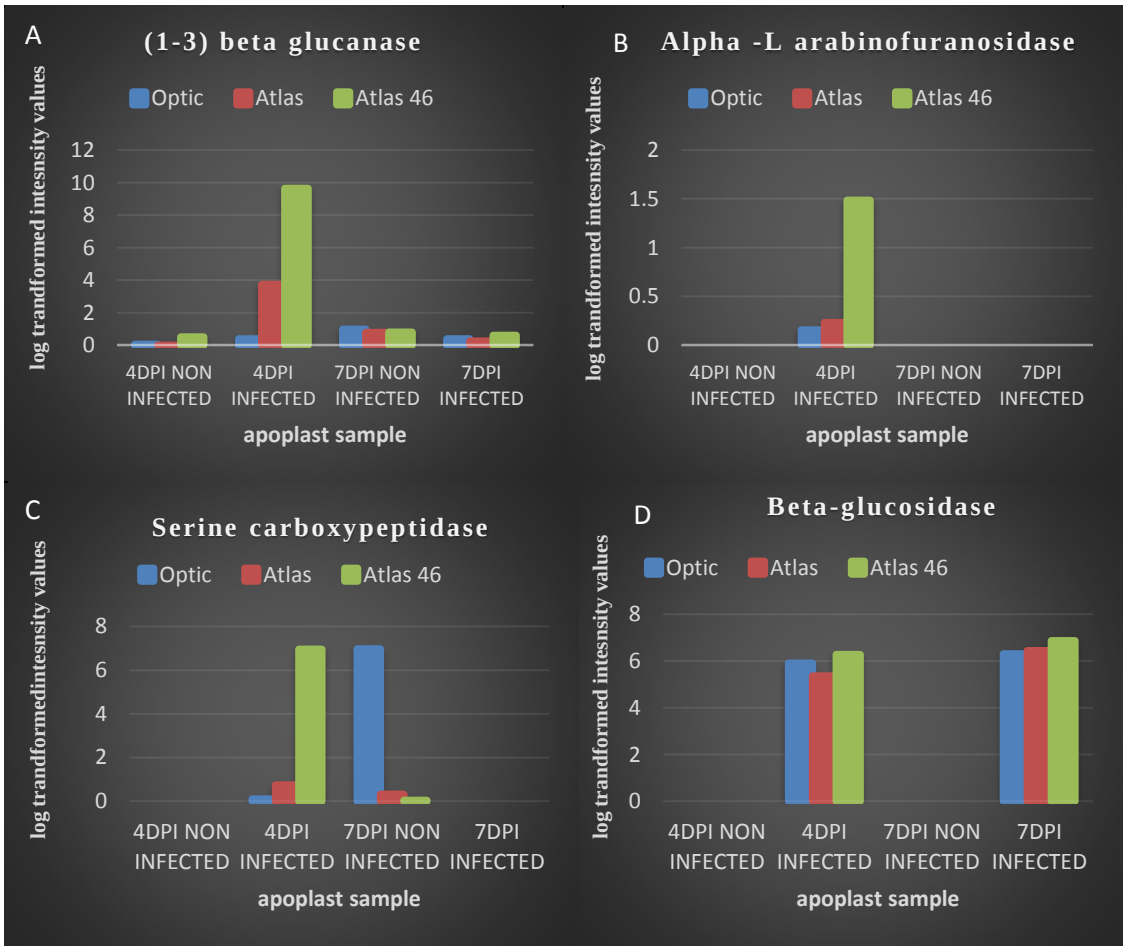


Figure 5.4: Proteins highly abundant at 4 dpi. Intensity values in apoplast samples from barley cultivars Optic, blue line, Atlas, red line, and Atlas 46, green line, from non-infected samples and infected samples (inoculated with *Rhynchosporium commune* strain 214-GFP) at 4 and 7 dpi. Samples are representative of one biological repetition.

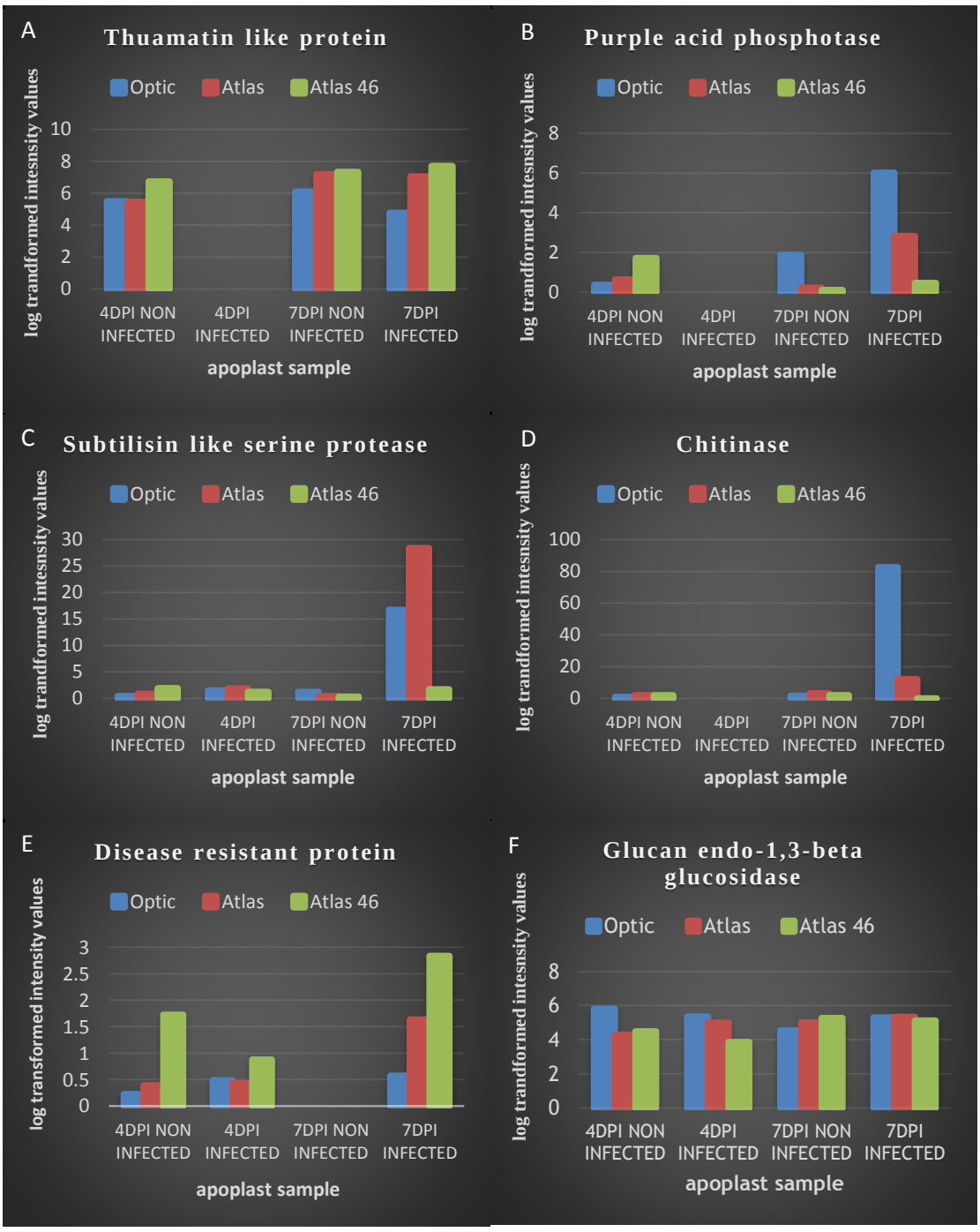


Figure 5. 5: Proteins highly abundant at 7 dpi. Intensity values in apoplast samples from cultivars Optic, blue line, Atlas, red line, and Atlas 46, green line, from non-infected samples and infected samples (inoculated with *Rhynchosporium commune* strain 214-GFP) at 4 and 7 dpi. Samples are representative of one biological repetition.

5.3 Discussion

Little is known about the intricate mechanisms that underpin a resistant response in the barley - *R. commune* pathosystem. To discover novel resistant barley lines, the first aim of this work was to identify new sources of resistance to *R. commune* using pathogen strains with different race specificities, and to provide further insight into a resistant response through the growth and morphology of the pathogen using confocal microscopy as has been described for *Rrs1* barley genotypes (Thirgnanasbanadam *et al*., 2011).

The pathogenicity test highlighted the high level of resistance conferred by *Rrs3*, *Rrs4* and *Rrs13* to the majority of *R. commune* strains, indicating the presence of corresponding Avr proteins in all those strains. However, highly virulent strains AU2 and L77 were able to overcome all 3 of these resistance genes. The ability of *R. commune* populations to rapidly evolve means that single *R* genes are not able to defeat the pathogen and plants are unable to evolve at the same rate which may be the reason in this case for barley susceptibility (McDonald *et al*., 2002)

Analysis of asymptomatic infection by *R. commune* GFP-expressing strain 214 helped to characterise its interaction with the barley line BC line 30 containing *Rrs13* as compatible, while restriction of its growth to the inoculum spot in leaves of line CI11549 containing *Rrs4* suggests that it is resistant to strain 214 and strain 214 should contain AvrRrs4 in addition to AvrRrs1. In addition, the response to *R. commune* infection by Syrian landraces SLB 66.024 shared a high level of similarity to Atlas 46, containing two resistance genes. Microscopic analysis revealed that a decreased fungal growth and random mycelial growth patterns were characteristic for the landrace, suggesting that it might contain an allele of *Rrs1* or another *R* gene recognising other avirulence gene(s) present in 214-GFP. Further genetic tests are required to investigate

this possibility, but the absence of diagnostic markers for *Rrs1* make proving that barley accessions contain *Rrs1* difficult.

The underlying reasons for restriction of growth and/or differences in colony morphology need to be investigated further. Hence, the extraction of proteins from the apoplastic fluid of infected plants is another approach to discover fundamental molecules which provide a more relevant biological representation of molecules associated with resistance.

The level of proteins potentially involved in resistance in most cases was as expected with high abundance in 4 and 7 dpi infected apoplast samples. The cell wall is an integral part of plants as it is a first line of defence against plant pathogens (Underwood, 2012). Proteins involved in plant cell wall modification, metabolism, and development were identified. This included an α-L-arabinofuranosidase involved in cell wall reorganisation which has been suggested as a putative defence related protein and was highly abundant in Atlas46 at 4dpi infected apoplast samples. The protein was compared in resistant and susceptible tomato plants infected with a virus and was shown to increase in expression by a 3-fold change in resistant plants (Chen *et al*., 2016). The protein has also been found in pathogens to aid with plant cell wall breakdown (Morant *et al*., 2008).

Pathogenesis related (PR) proteins which are well known to participate in complex plant defence responses to pathogens were also identified. Glucan endo-1,3-beta-glucosidase- PR2 playing a role in the hydrolysis of fungal cell walls (Chatterjee *et al.*, 2014)). The upregulation of the protein in the secreted fraction of *Oryza meyeriana* cultured cell suspension after 24 hours after inoculation Xanthomonas oryzae pv. oryzae highlighted its importance as a defence protein (Chen *et al*., 2016). In addition chitinases are well characterised enzymes that break down fungal cell walls. Chitinase

was more abundant in Optic than in the resistant cultivar Atlas 46, however by analysing the 214-GFP images of infection the lack of growth in the apoplast may be the reason for the low abundance. However, the results are representative of only one biological repetition hence, further validation would be required. In addition, thaumatin-like protein was expected to be expressed earlier in the infection; however it was highly abundant at 7 dpi. Thaumatin like proteins are from the large family PR5 family and are also active antifungal agents (Vigers *et al*., 1992). Over-expression of a thaumatin protein in transgenic rice enhanced resistance to the soil borne plant pathogen *Ralstonia solani* (Dalta *et al*., 1999). A Serine carboxypeptidase –identified in the BRS1 cell surface receptor for brassinosteriods was also revealed and again was found at high levels at 4dpi in Atlas46. The upregulation of the cell surface receptor in rice was identified and constitutive expression of the protein in transgenic plants increase tolerance to oxidative stress (Liu *et al*., 2008). One Subtilisin like protease was present and is known to accumulate in viroid infected plants (Tornero, 1995). Purple acid phosphatases are able to generate ROS against bacterial pathogens in Arabidopsis and under prolonged P starvation are required for basal resistance to *P. syringe* (Ravichandran *et al*., 2013) and a disease resistant protein. Although the extraction of the apoplast is relatively laborious and in some cases protein identification can be limited, this work has identified some important plant molecules that could be further analysed with the potential use of markers to barley resistance to *R. commune*.

CHAPTER 6

6. Characterisation of *Rhynchosporium commune* interaction on the non-host plant *Nicotiana benthamiana*

6.1 Introduction

Understandably, most of the research to date has focused on the narrow host range of *R. commune* due to the damage it causes as a pathogen (Zhan *et al*., 2008). Interestingly, a recent study conducted by King *et al*., (2013) identified *R. commune* pathogenic on Italian ryegrass, which was not previously classified as a host. In addition, as seen in Chapter 5, *R. commune* has the ability to grow and survive asymptomatically on its host barley. This raises questions to whether about the pathogen can infect or survive on any other plant species. Previous preliminary research suggested that *R. commune* spores could indeed germinate and produce mycelium after inoculation with GFP expressing isolate 214 on the non-host, model plant *Nicotiana benthamiana* (Avrova, unpublished data). No other research has been conducted on the growth of *R. commune* on alternative plants since.

From a phytopathological perspective, plant species on which disease symptoms have not been observed, are considered to be non-hosts for a pathogen (Malcom *et al*., 2012). In agriculture, growers aim to eliminate a microorganism only if they are known as a pathogen. Consequently, the broader repertoires of ecological interactions of this pathogen have not been investigated and knowledge in this area is extremely limited.

To survive intercrop periods or unfavourable conditions, most plant pathogens possess mechanisms such as an epiphytic, saprophytic or resting phase. *R. commune* has been identified on infected seed but it has also been proposed that the pathogen has a saprophytic phase allowing it to survive during intercrop periods on plant debris (Shipton et al., 1974). However, there has been no research conducted to assess if *R. commune* may in fact survive on alternative hosts as a potential epiphyte, even considering the length of time it can survive undetected. During an epiphytic phase of the lifecycle, the pathogen survives on the surface of their host in a non-parasitic relationship. It is not uncommon for plant pathogens to adopt contrasting lifestyles, by completing their life-cycle as pathogens on some hosts, while living as commensals or mutualists on others (Schulz and Boyle, 2005; Malcolm *et al*., 2013). In addition, microorganisms can also display different lifestyles within a single host. (Casadevall and Pirofski, 2003; Schulz and Boyle, 2005; Newton *et al*., 2010). It is possible that *R. commune* may survive on other plant species which have been termed non-hosts without producing symptoms (Casadevall, 2007; Giauque and Hawkes, 2013; Iliev and Underhill, 2013).

As seen throughout this research, the development of a GFP expressing *R. commune* isolate has been a valuable tool for understanding the mechanisms of the pathogen's growth during infection (Linsell *et al.*, 2010) and in response to barley *Rrs1* genotypes (Thirugnanasambandam *et al*., 2010). In general, fluorescent confocal microscopy has facilitated the exploration of pathogen growth during infection on hosts and has provided opportunities for elucidating pathogen molecules that function in pathogenesis (Harham, 2012). The main advantage of using confocal microscopy is the narrow depth of field and that it permits deep sample visualisation within living tissues and cell. In the past 20 years GFP has been successfully used as a reporter and vital marker in many

prokaryotic and eukaryotic systems (Zimmer, 2002). The ability to generate highly visible, real time images of pathogen infection is not only intrinsically fascinating but advantageous for plant pathologists.

Many dicotyledonous plants like *N. benthamiana* have now been used for many years as model plants within the laboratory (Goodin *et al*., 2012). *N. benthamiana* is highly susceptible to many strains of oomycete and fungal species allowing for the analysis of pathogen during infection (Goodin *et al*., 2008). Furthermore, the use of *N. benthamiana* as a model species has played a major role in furthering detailed understanding of the functional characterisation of plant pathogen effectors and the mechanisms of non-host resistance (Petre *et al* 2006; Faino *et al*., 2008; Stem *et al*., 2014; Becktell *et al*., 2014). Due to the high efficiency rates of genetic transformation, coupled with extensive sets of technical resources and databases, makes this plant species a popular model plant for plant pathology research (Goodin *et al*., 2015). As *N. benthamiana* is classified as a non-host for *R. commune*, information into the interaction of *R. commune* and *N. benthamiana* will provide insight into potential non-host resistance. In addition, obtaining further information of the lifestyle of *R. commune* on alternative plant species may aid in a better strategy to control this pathogen.

6.2 Results

6.2.1 Asymptomatic growth of *R. commune* does occur on the model plant species *N. benthamiana*

To further investigate the possibility of *R. co*mmune growth on other plant species with the absence of any visual disease symptoms, *R. commune* inoculations were carried out on the model plant species *N. benthamiana*. Drop inoculations of spores from *R. commune* strain 214-GFP was carried out on leaves of *N. benthamiana* plants.

N. benthamiana plants with plasma membrane protein tagged with a red fluorscent protein were used to determine if any signs of damage were occurring inside the leaf tissue. At 5dpi, microscopic anlaysis of *R. commune* revealed the germination of fungal conidia. (Figure. 6.1 A & B). By 9 dpi fungal mycelium had started to develop and the growth of the fungus from the original inoculation spot had increased (Figure 6.1 C & D). At 15dpi there was a noticable increase in the amount of mycelium (Figure. 6.2 A & B). From this point and until the last day of analysis the fungal mycelium did not grow in the same manner as it would on its host barley, outlining the epidermal cells. In fact the growth resembled that of an incompatible infection on barley, explorative hyphae growing in all directions (Thirugnanasambandam *et al*., 2010). The spread of the fungus did persist over time resulting in a sizeable colony by 28 dpi (Figure 6.3 A-C). There were also some possible signs of sporulation at 28 dpi (Figure 6.3 C). The plant showed no evidence of plasma membrane deterioration, as would be seen during the late stages of infection in barley. The plant plasma membrane was unimpaired which was clearly evident at the later time point, 28dpi (Figure 6.3 C). Throughout the entire experiment no macroscopic signs of infection were visible (Figure 6.4 A & B).

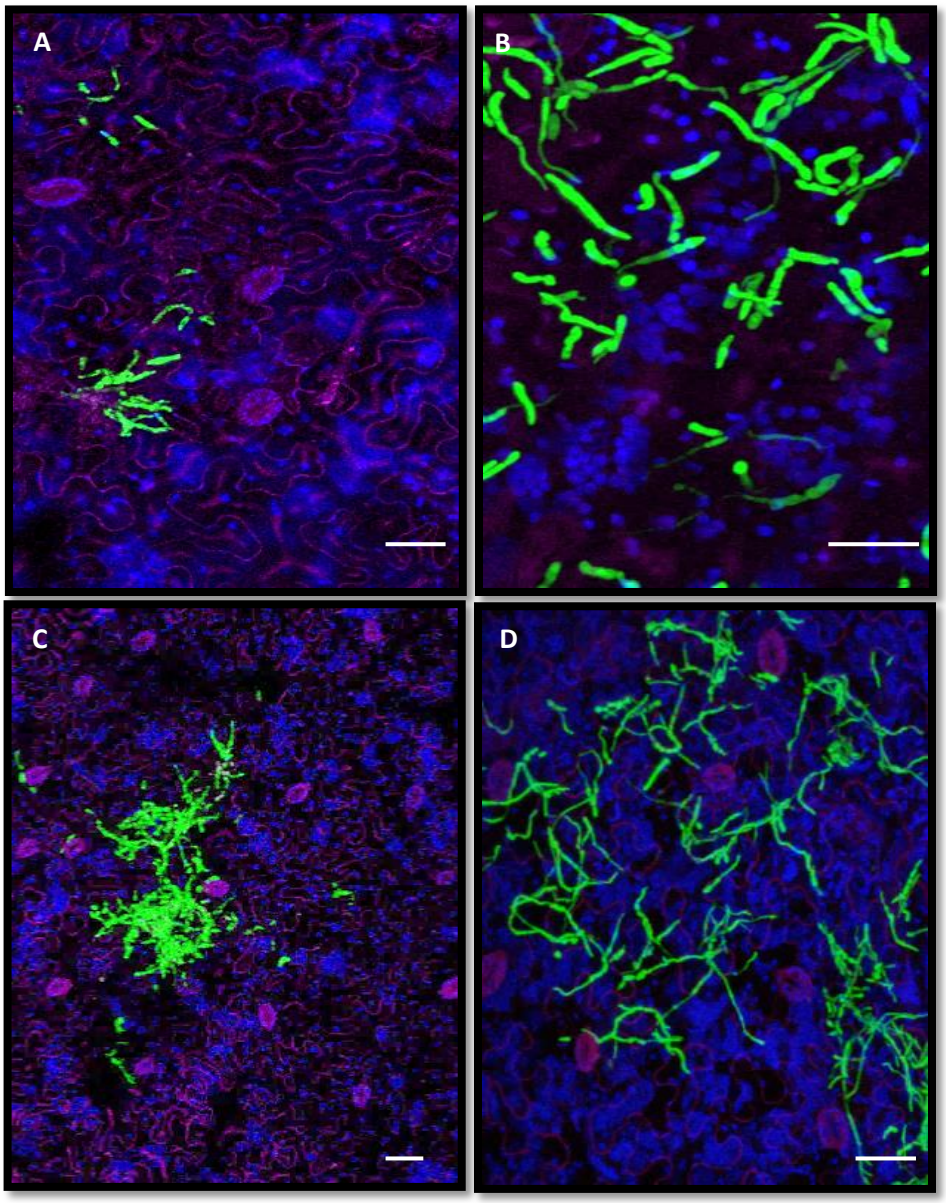


Figure 6.1: Confocal microscopy images of *Rhynchosporium commune* strain 214-GFP on *Nicotiana benthamiana* line CB173 expressing red plasma membrane marker A) 5 dpi germinating conidia B) higher magnification of germinating conidia at 5 dpi C) 9 dpi mycelial growth D) higher magnification of mycelial growth at 9 dpi. 214-GFP strain excitation of 488 nm and emission collection of 500-530 nm. Plant plasma membrane marker DsRed with an excitation 525 of and emission collection of 550-575nm. At the same time the autofluorescence signal from plant chlorophyll was collected with an emission range of 650-700 nm. Scale bars = 50µm

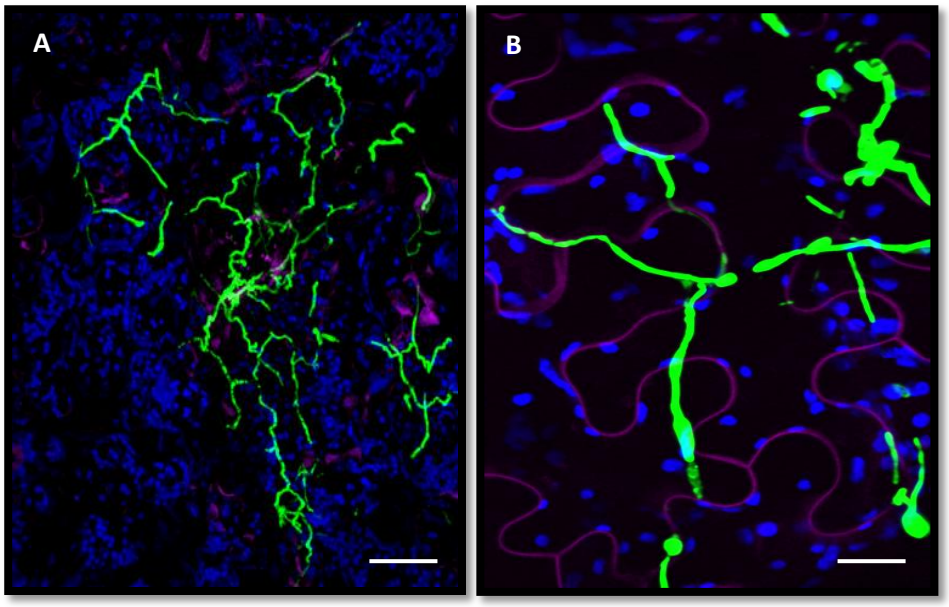


Figure 6.2: Confocal microscopy images of *Rhynchosporium commune* strain 214-GFP on *Nicotiana benthamiana* line CB173 expressing red plasma membrane marker at 15 dpi. 214-GFP strain excitation of 488 nm and emission collection of 500-530 nm. Plant plasma membrane marker DsRed with an excitation 525 of and emission collection of 550-575nm. At the same time the autofluorescence signal from plant chlorophyll was collected with an emission range of 650-700 nm. Scale bars = 50µm

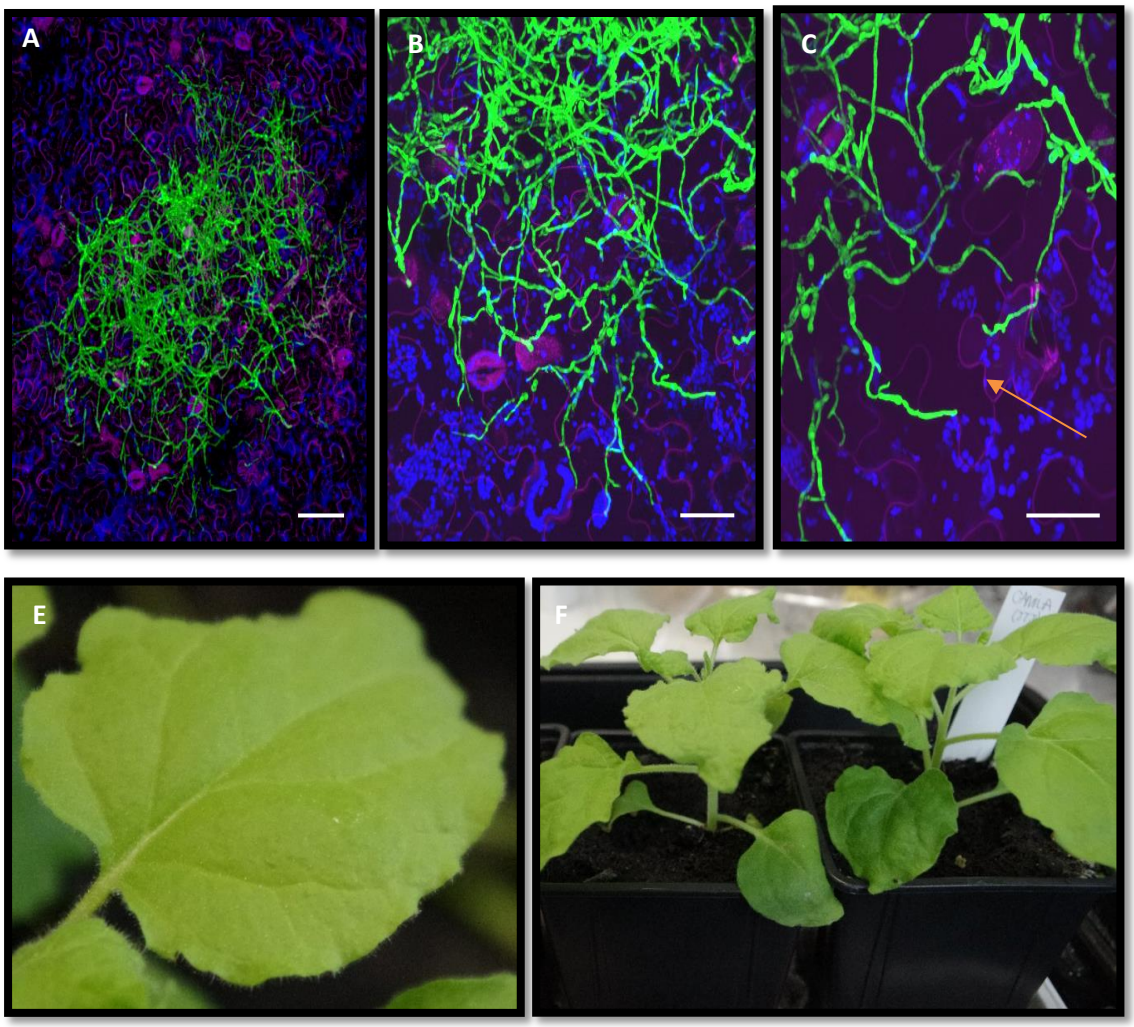


Figure 6.3: A-C Confocal microscopy images of *Rhynchosporium commune* strain 214-GFP on *N. benthamiana* line CB173 expressing red plasma membrane at 28 dpi . Orange arrow shows intact plant plasma membrane. *N. benthamiana* plants inoculated with *R. commune* strain 214-GFP at 9dpi and E) 28dpi, showing no macroscopic symptoms. 214-GFP strain excitation of 488 nm and emission collection of 500-530 nm. Plant plasma membrane marker DsRed with an excitation 525 of and emission collection of 550-575nm. At the same time the autofluorescence signal from plant chlorophyll was collected with an emission range of 650-700 nm. Scale bars = 25µm

6.2.3 Growth of *R. commune* strain 214-GFP on non-plant surfaces

It may be possible that during the collection of conidia from culture plates that despite the attempts to remove all media, some may remain and could potentially increase the chance of *R. commune* being able to grow on the plant leaf without access to the apoplast. In order to determine if the growth of *R. commune* on a non-host was a valid result, *R. commune* spores were drop inoculated onto two different types of material – glass microscope slide and plastic Petri dish and treated in the same experimental way as an inoculated plant. At two different time points the samples were analysed under the confocal microscope. At 5 dpi, the spores were viable and some germination had begun on both surfaces (Figure 6.5 A & C). There were also some visible signs of mycelium present (Figure 6.5 A) – likely to be fragments that escaped retention during conidial isolation. At the second-time point, 28dpi spores were still detected although the concentration appeared lower and there was a lower emission of fluorescence and no presence of any germinating conidia (Figure 6.5 B & D). In addition, there was no presence of mycelial fragments from the first-time point, indicating the inability of *R. commune* to continue growth on both surfaces.

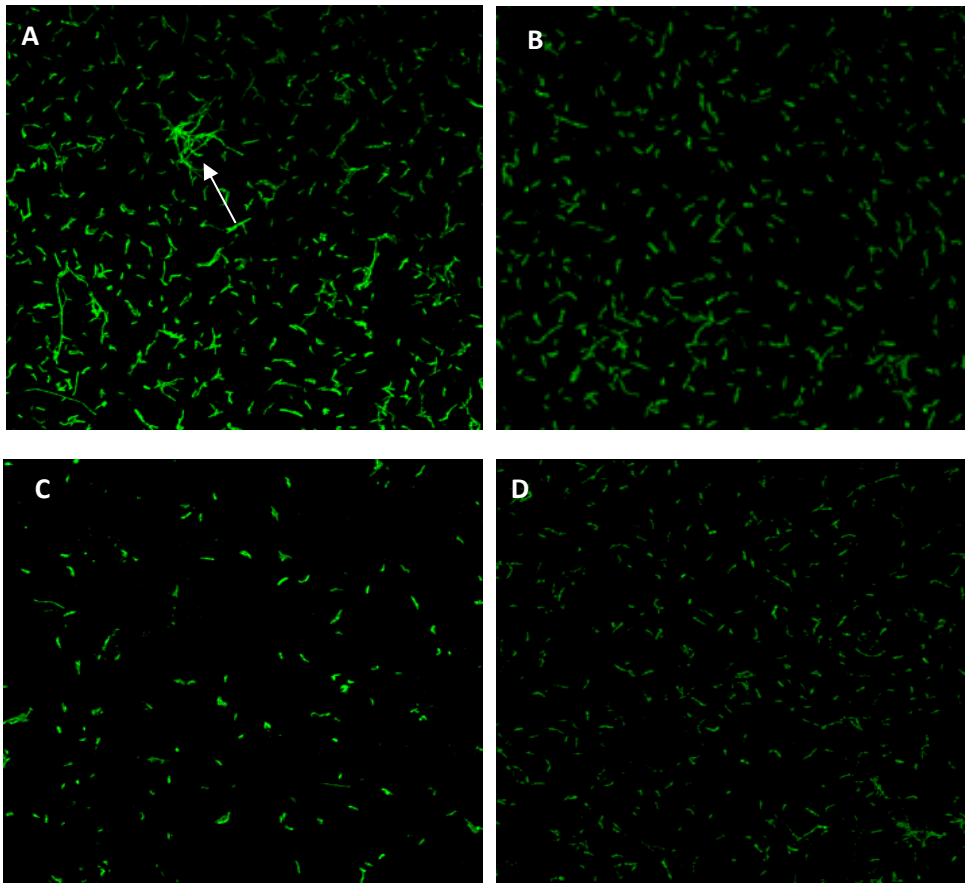


Figure 6.4 Confocal image of *R. commune* strain 214-GFP spores on two different surfaces: on plastic at A) 5 dpi and B) at 28 dpi; and on glass slide C) at 5 dpi and D) at 28 dpi. White arrow showing mycelial fragments. Scale bars = 50µm

6.2.4 *R. commune* gene expression during growth on non-host *N benthamiana*

6.2.4.1 Analysis of NIP expression on non-host *N benthamiana*

Microscopy has revealed the ability of *R. commune* to survive on a non-host, however it does not provide any information regarding the nature of the plant-fungal association. If *R. commune* has the ability to grow on a non-host, the question remains if it is able to recognise the non-host and if it expresses effectors that are required for pathogenesis.

Pathogen effectors are known to be highly up regulated during infection on host plants (Jonge *et al*., 2011; Zhu *et al*., 2013). *R. commune* NIP1 and NIP3 are thought to have a role in nutrient acquisition, thus investigation of the expression of the genes during the growth on *N. benthamiana* should provide some insight (Wevelsiep *et al*., 1991). The NIP genes were only upregulated at the first two time points of interaction. NIP1 was the most highly abundant with high expression at 1dpi but four times that amount at 2dpi, reaching 3 times the level of the endogenous control Actin. On the other hand, NIP2 and NIP3 showed higher abundance at 1dpi and lower at 2 dpi. At 1 dpi NIP2 was over 30% and NIP3 was the least abundant of all NIPs at around 1 % the amount of NIP1.

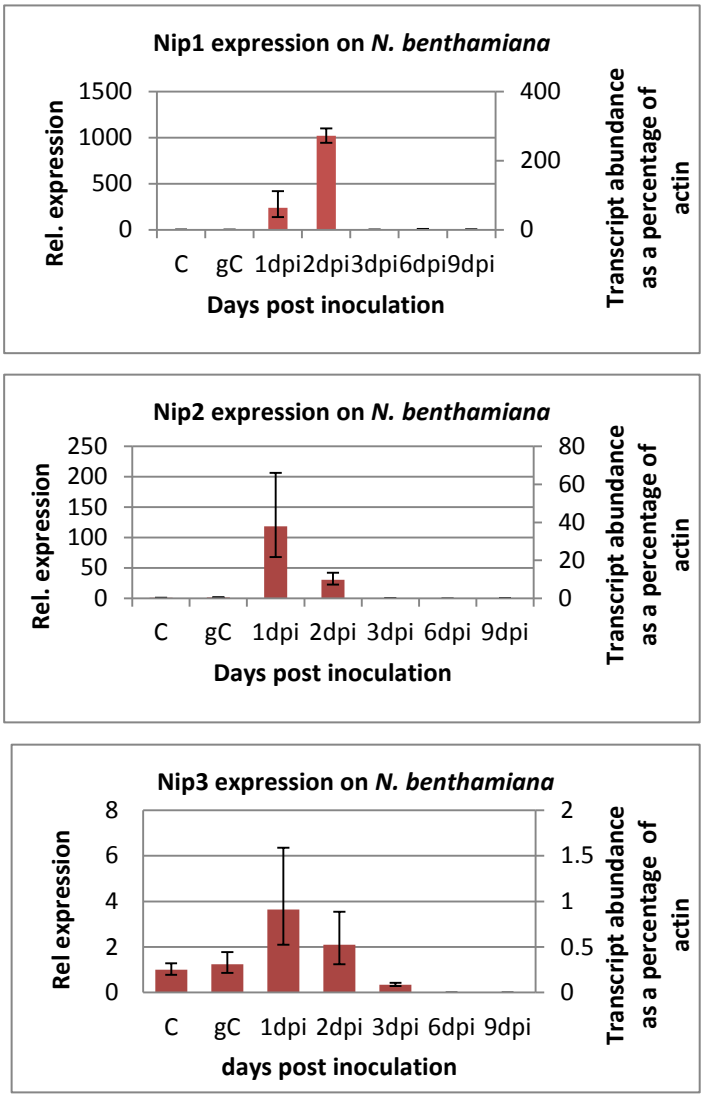


Figure 6.5: Relative expression of A) NIP1, B) NIP2 & C) NIP3 in *R. commune* strain 214-GFP during its growth on *N. benthamiana*. Bars indicate confidence intervals calculated using three technical repetitions.

6.2.4.2 Analysis of expression during growth on *N. benthamiana* leaves of the *R. commune* effector *RcINS1* inducing cell death in *N. benthamiana.*

RcINS1 is highly upregulated in barley infection, similar to the NIPS. In addition, recent studies revealed that it is recognised in the apoplast of *N. benthamiana* and expression within the plant causes cell death. However, as shown *R. commune* shows no signs of infection or no cell death when grown on *N. benthamiana*. Therefore, it was of interest to determine the expression of this effector during growth if the pathogen. The expression profile revealed that at 1 dpi the gene is upregulated to the level similar to that in barley at the same time point corresponding to initial conidia germination. However, while *RcINS1* transcript abundance continues increasing during barley colonisation reaching 40-50 times the level in conidia by 3 dpi before declining back to original level the expression during *R. commune* growth on *N. benthamiana* leaf drops of at 2 dpi but is then increasing again slowly from 3 to 6 dpi to the level similar to that at 1 dpi before dropping off again by 9 dpi.

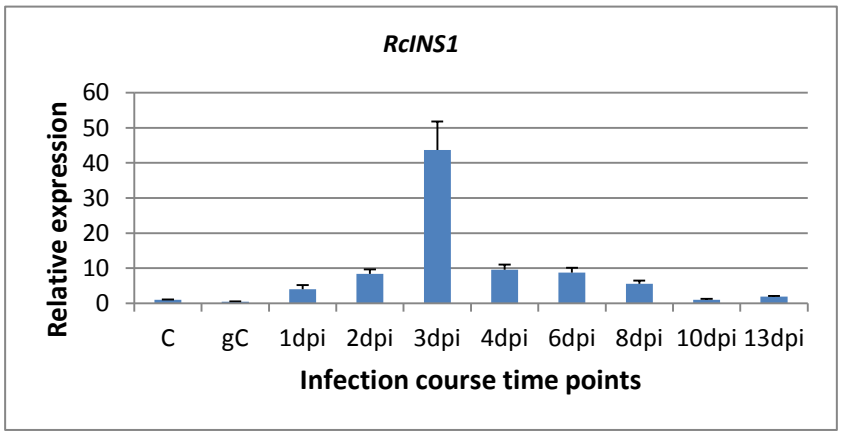


Figure 6.6: Relative expression of *RcINS1* in *R. commune* strain L2A during infection on barley. Bars indicate confidence intervals calculated using three technical repetitions.

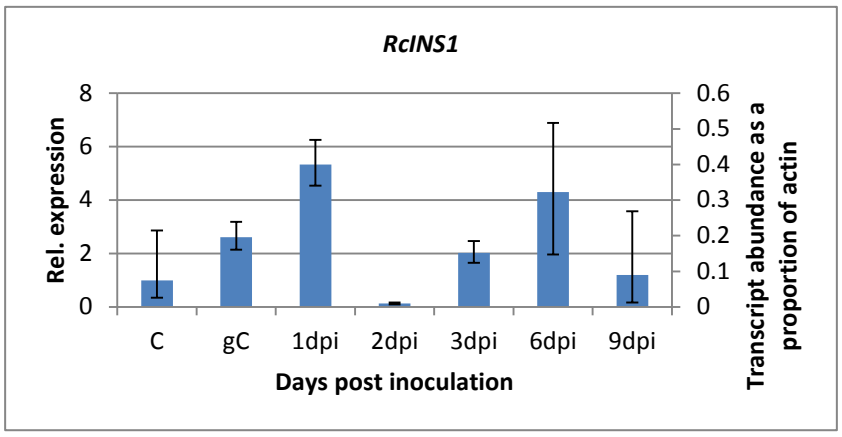


Figure 6.7: Relative expression of *RcINS1* in *R. commune* strain 214-GFP during its growth on *N. benthamiana*. Bars indicate confidence intervals calculated using three technical repetitions.

6.3 Discussion

The development of recombinant fluorescent probes and advances in microscopy technologies have revolutionised the study of plant–pathogen interactions (Hickey *et al*., 2005). Without the ability to visualise *R. commune* growth on plant species it would have not been possible to investigate potential growth of the pathogen on a non-host and further analyse the interaction of *R. commune* with *N. benthamiana*.

The results showed that the fungus was able to germinate and produce mycelium after inoculation with conidia of the leaves of *N. benthamiana*. The lack of growth on non-plant surface provided strong evidence that the fungal growth was not due to nutrients obtained from media traces. The viability of the spores on non-host surfaces indicates that *R. commune* spores are able to survive as resting structures. The production of robust spores as part of a pathogens lifecycle is a common mechanism. (Brown & Hovmøller, 2002).

It was surprising to see that *R. commune* was still viable at 28 dpi. However, the growth was not as extensive as it would be in a compatible interaction with its host barley (Thirugnanasambandam *et al*., 2010). In addition, analysis of the plant plasma membrane revealed no deterioration, suggesting that pathogen was unable to penetrate the plant cuticle to gain access to the plant apoplast. This was further backed up by the lack of expression of the three NIPs which are proposed to stimulate the plasma membrane H+ATPase during nutrient acquisition leading to the collapse of the epidermal tissue (Wevelsiep *et al*., 1991). In addition, the lack of substantial *RcINS1* upregulation also confirmed the absence of the fungus in the apoplast, as the effector is known to elicit a plant cell death response. The expression of effectors at early time points and the lack of mycelial growth on non-plant surfaces may indicate the ability of the fungal spores to recognise plant surfaces (Braun, & Howard., 1994). It would have been of added benefit to assess the expression of effectors on non-plant surfaces to provide further evidence of this.

The interaction of non-host plants and important crop pathogens is now receiving more attention due to the potential transfer of NHR genes to create potentially longer lasting resistance (Lee *et al*., 2016). In the case of this research it appears that it is possible that non host resistance of *N. benthamiana* to *R. commune* is associated with preformed barriers such as surface waxes of the plant leaf cuticle (Tsuba *et al*., 2002; Uppalapati *et al*., 2012; Gill *et al*., 2015). It is also possible that non-host resistance acts at the time of penetration (Hoogkamp *et al*., 1998; Trujillo *et al*., 2004; Zellerhoff *et al*., 2006). This mode of defence has been well described in barley non-host resistance to various fungal pathogens (Zellerhoff *et al*., 2010). However, a cell death response is usually associated with this phase of non-host resistance (Mysore & Ryu, 2004). It has been suggested that PTI is the major obstacle that pathogens need to overcome as the evolutionary distance

between host and non-host increases (Lefert & Panstruga, 2011). On the other hand, the research indicates that the pathogen may be able to survive on alternative plant species as an epiphyte.

This research has given a first insight into the growth of a fungal pathogen of grasses on a dicotyledonous plant species. Without the ability to penetrate the cuticle of *N. benthamiana,* it is not likely that *R. commune* pathogenesis could be researched in this model organism. However, there is possibility to explore the mechanism of non-host resistance to *R. commune* using *N. benthamiana* as a model plant, but there is still much research that needs to be accumulated. Furthermore, the source of primary inoculum on crops is fundamental to management of agriculturally important diseases. To determine whether *R. commune* has the ability to survive on alternative crop species throughout the growing season will rely on larger based field research studies to confirm this theory. However, if this is the case, further insight may increase the capacity to manage the disease.

CHAPTER 7

7 General discussion

As the global population increases rapidly, agriculture struggles to maintain the levels of crop production required for the immense rise in food demands. Plant pathogens have a high capacity to produce substantial disease levels on food crops, reducing the production and quality of food. Hence, greater emphasis to reduce the impact of crop disease is required. In many cases chemical treatment to limit or eradicate diseases are used, however the environmental impact of the applications can result in consequences to non-target organisms, result in pesticide drift and residues on food (Kilbrew & Wolff, 2010). Agriculture is faced with the challenge to maximise crop yields while decreasing negative environmental impacts. However, several factors influence the reduction of food security imposed by pathogens. The lack of well-developed diagnostic tools to identify asymptomatic pathogen infection can lead to severe disease implications later in the growing season. In addition, the level of disease severity can be overlooked due to subjective rather than quantitative methods to detect pathogen biomass accumulation. Furthermore, experimental obstacles preventing the mapping and cloning of plant resistant genes in conjunction with the variation and vast amounts of evolving pathogen molecules, results in the lack of complete understanding of the mechanisms of resistance and pathogen infection. Therefore, the development of methods to identify pathogens, experimental research to gain an understanding of pathogen effectors, how the pathogen infects and the molecules involved in plant defence against pathogens will result in better understanding of how we can improve methods for diagnostics and predicting crop durability.

As pathogens are known to use effector molecules to overcome plant resistance (Dangl & Jones, 2006), this study began with the exploitation of the genome and transcriptome sequences of *R. commune* to identify novel candidate effectors. Revealing pathogen effector function provides insight into the infection progress. The importance of effector discovery is high as the research into *R. commune* effector repertoire is still in its infancy. Rapid identification was hindered by the lack of effector-specific motifs. The absence of effector-specific motifs is probably a consequence of secretion into the apoplast instead of effector translocation into the host plant cells as that is what the RXLR motifs present in *P. infestans* effectors is required for (Whisson *et al*., 2007). However, all predicted candidates contained characteristic features common of other apoplastic effectors. However, more recent studies are beginning to include other lines of evidence associated with fungal effectors and are potentially powerful for predicting effector candidates. Saunders *et al*., (2015) developed a pipeline for hierarchical clustering to classify and rank candidate effectors of rust. Combination of additional lines of evidence such as diversifying selection and selection of effector candidates not found in non-pathogenic strains have been used to prioritise candidate sequences (Syme *et al*., 2013; Sperschneider *et al*., 2014). Overall, there is scope for the accumulation of further effector characteristics if further research into the biochemical properties of effector proteins is conducted.

The pathogen expression profiling was effective in determining the timing and levels of gene expression and can be used to indicate the involvement of specific genes in pathogenesis. qRT-PCR is the most sensitive and specific method of transcription quantification allowing to detect less abundant transcripts at much earlier time points during the infection. This was a necessity for detecting mRNAs from *R. commune* at the early stages of infection as fungal biomass would be low. Upregulation during

infection was a useful characteristic to prioritise the candidates and allowed to reveal novel candidate effectors highly abundant during the biotrophic stage of interaction.

Extremely low efficiency of targeted gene disruption in *R. commune* limited the possibility of functional characterisation. However new technologies using CRISPR Cas9 technology are now being developed in fungi. This application is becoming more popular due to the efficiency in gene editing (Matsurura *et al*., 2015). In addition, over expression may have provided information if any of the effectors increased the pathogenicity of the transformed isolate. However, unlike *P. infestans* where this method has been used efficiently (Boevink *et al*., 2016) the growth of *R. commune* is relatively slow and therefore may not give a clear indication of effector function. Additionally, expression of effectors is finely tuned during infection *in planta* and increasing the level of expression would not necessarily be beneficial to the pathogen.

Secretion into the apoplast or cytoplasm from the pathogen cell is fundamental for the effector to carry out its virulence function. The examples of experimental continuity from gene identification and quantification of transcript abundance during infection to the detection of the protein during plant infection are limited in the literature. This is mainly due to the difficulties of isolating proteins which are likely to be far less abundant than plant proteins. The low quantity of fungal proteins identified in chapter 3 is an example of such, where the accumulation of plant proteins within the sample has likely masked the low expressed proteins. In addition, difficulties in obtaining apoplastic fluid from infected material has been documented (Nouchi *et al*., 2010). Despite this, the apoplastic fluid extraction conducted in this project identified novel candidate effectors which can be prioritised for future research. The identification of RcLysM3 was an important discovery, indicating its high abundance within the

apoplast. Further characterisation revealing chitin binding abilities and avirulence correlation indicates an essential nature for this protein during pathogen survival in the apoplast. Further research is now required to show evidence of this. Silencing and CRISPR-Cas9 constructs have recently been generated for this effector to enable functional characterisation through the interference of the regulation of gene expression and gene targeting, respectively. Another possible route to confirm its function could be complementation of the Mg3LysM knock out mutant line of *Z. tritici* which is also available (Marshall *et al*., 2012). However, there is the potential for the effector not to be essential due to the amount of LysM domain sequences within the *R. commune* genome suggesting a possibility of functional redundancy. It is reasonable to suggest that the fungus deploys a range of chitin-binding effectors due to its location during infection and the consequent accumulation of chitin within the apoplast. Therefore, using polysaccharide affinity assays to determine the binding ability of other LysM domain proteins will be a starting point, followed by single or multiple gene silencing or knockouts.

Barley R gene resistance to *R. commune* has not proved durable. Revealing that effectors are essential for pathogenicity and potentially recognised by the host plant (Avr genes) is an important factor. Essential effectors are less likely to be deleted or altered by the pathogen and subsequently the ability of the pathogen to evade the recognition of the plant resistance protein decreases. Therefore, the discovery of novel avirulence genes that are required for pathogenicity is a critical step to identify more durable forms of resistance to this devastating fungal disease.

Despite the identification of AvrRrs*1* recognition by the *Rrs1* 25 years ago, there is very little information on the intricate molecular mechanisms that occur in a resistant

response. To identify novel resistance to this pathogen cultivar were selected containing different *R* genes to that of *Rrs1*. The virulence teasing approach helped to prioritise barley lines for further analysis using the 214-GFP strain. Microscopic assessment of the extent of the growth and the colony morphology were used to distinguish between susceptibility and potential resistance to *R. commune*. This is one of the characteristics of *Rrs1* that has been previously highlighted (Thirugnanasambandam *et al*., 2010) Although barley lines presenting no symptoms and a decrease in biomass, physically restricted growth and / or random colony morphology could be a sign of resistant interaction, it still remains difficult to determine the durability of the plant defence. It is possible that some *R. commune* strains develop much slower throughout the growing season but the accumulation may have an impact on the crop yield although no research has looked into this possibility. In addition, a range of *R. commune* strains need to be used to distinguish the level of asymptomatic infection. The production of some other fungal strains expressing fluorescent proteins would be highly beneficial for future research, especially for highly virulent stains such as AU2. Partial resistance could also be potentially at play, as it is also characterised by reduced growth of the pathogen. Again, there is a need to gain a better understanding of this type of resistance.

Due to the lack of evidence to allow full confirmation of resistant lines, a proteome approach to identify the key players in *Rrs1* resistance was conducted. Initial research began on the contents of apoplast and its importance in plant pathogen interactions was identified almost 30 years ago. However only a few studies have focused on plant-pathogen interactions in the apoplast (Mehta *et al*., 2008).

The identification of PR proteins such as thaumatin-like protein and β- glucanase present in all of the cultivars used indicated similar components of basal defence

mechanism. In addition, the PR proteins have been identified in numerous studies of the upregulation of PTI (Steiner-Lange *et al*., 2003). However, the abundance in the resistant line was slightly higher. Only the disease related protein and α-L arabinofuriodase were highly upregulated in comparison to Optic and Atlas suggesting a specific role in the *Rrs1*-controlled resistance. It is possible that some proteins which were down regulated in Atlas 46 may be due to the protein being a susceptibility factor for disease and is not upregulated in a resistant response. Only one biological repetition was available for analysis due to the inefficient labelling, other repetitions would be required to provide rigidity to the results.

Both *R. commune* and barley protein databases were available to upload to the MaxQuant software allowing the identification of proteins in the apoplast. Inevitably the annotation of the genome sequences of *R. commune* and barley are not complete, hence here may be many additional proteins that were not identified on this occasion. In addition, not all genetic elements of the genome have been annotated and many proteins identified in this work were hypothetical proteins. Furthermore, the use of MaxQuant for protein identification is limited to the sequences present in the manually inserted databases, therefore if the sequences are not present they will not be identified. In addition, the peptide identification in MaxQuant is limited to sequence with no scope for the variation that may occur in sequences due to mutations. In general, this decreases the chances of identifying other interesting candidates of disease resistance.

Major *R* gene resistance is still heavily relied on in agriculture systems to protect against crop disease, although protection against serval strains of a pathogen may be incompletely effective. However, the use of *R* gene pyramids may provide an alternative and more effective strategy to control various *R. commune* pathotypes. (Zhan

et al., 2012). More recently the investigation of non-host resistance has become more prominent in the literature. Non-host resistant is only beginning to be understood but in contrast to major *R* gene resistance the response involves multiple pathways (Gill *et al*., 2015) and is known to provide resistance to many pathogens. Despite the inability to use *N. benthamiana* as a model organism for experimental research of *R. commune* infection process, the results have indicated this plant species as a non-host. It is highly possible that sources of non-host resistance can be identified and elucidated and effectively transferred in future plant breeding. However, further investigation of possible epiphytic growth on *N. benthamiana* is needed to determine the type interaction occurring. This may include the inoculation of other model dicotyledonous species such as *Arabidopsis thaliana* and potentially, crops that are used in rotation with barley such as oil seed rape (*Brassica napus).*

This research has provided a better understanding of *R. commune* pathogenicity, barley resistance mechanisms and an insight into non-host resistance to this pathogen. A wide range of techniques were used which could be further developed for future research and potentially diagnostic tools. Future research will benefit from the results obtained throughout the research chapters.

CHAPTER 8

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9. Appendix

9.1 Liquid and agar media

9.1.2 CZV8CM agar

Oxoid Czapek Dox	56.0g
Agar	10g
V8 juice	200 mL
Calcium carbonate	4.0g
Complete supplement	50 mL

Made up to 1 L with distilled water and autoclaved for 15 mins at 121°C

9.1.2 Potato Dextrose Broth / Agar (PDB /PDA)

Dextrose	20g
Potato starch	4g
Agar	15g

Made up to 1 L with distilled water, boiled whilst mixing and autoclaved 15 mins at 121°C.

9.1.3 Yeast Peptone Dextrose (YPD)

BactoYeast extract	4g
Bacto Peptone	8g
Dextrose	8g
For agar plates: Agar	8g

Made up to 400mL with distilled water and autoclaved 15 mins at 121°C

9.1.4 YNB + glucose

YNB with amino acids	5.36g
Glucose	2g

For agar plates:

Agar	15g

Made up to 400 mL with distilled water and autoclaved 15 mins at 121°C

9.1.5 Luria Broth and Agar plates (LB)

Bacto-tryptone	10g
Yeast extract.	5g
NaCl	10g

For agar plates:

Agar

15g

Made up to 800mL of distilled water and adjusted pH to 7.5 with NaOH. Volume adjusted to 1L with dH2O and autoclave for 15 mins at 121°C

9.1.6. Synthetic complete minus uracil (SC-Ura)

Yeast nitrogen base –aa	2.68g
Glucose	8g
Drop-out mix minus uracil	0.77g

For agar plates:

Agar	8g

Made up to 400 mL with distilled water and autoclaved for 15 mins at 121°C

9.1.7 Low salt Luria broth (LB)

tryptone	4g
yeast extract	2g
5M NaCl	7.84mL

0.4M NaOH (pH to 7.5)	4mL

Made up to 400 mL with distilled water and autoclaved for 15 mins at 121°C

9.1.8 SOC media

Bacto Tryptone	20g
Bacto Yeast Extract	5g
5M NaCl.	2mL
1M KCl.	2.5mL
1M MgCl2	10mL
1M MgSO4	10mL
1M glucose	20mL

Made up to 1 L with distilled water and autoclaved for 15 mins at 121°C

9.2 Lacto phenol trypan blue solution

9.2.1 Staining solution

Lactic acid	10mL
Glycerol	10 mL
Phenol	10 g
Trypan blue	10 mg
Distilled water	10 mL

9.2.2. De-staining solution

Dissolve 2.5 gm of chloral hydrate in 1 mL of SDW

9.3 Solutions and reagents for transformation

9.3.1 Protoplast preparation

9.3.1.1. KC solution

Final concentrations	200 mL	400 mL	800 mL	1000 mL
0.64 M KCl	9.54 g	19.08 g	38.16 g	47.7 g
0.2 M CaCl2	5.88 g	11.76 g	23.52 g	29.4 g

Dissolved in molecular biology grade water, dispensed into 100 mL aliquots, autoclaved to sterilize.

9.3.1.2. MT solution

Final concentrations	200 mL	400 mL
1 M mannitol	36.43 g	72.86 g
10 mM Tris.HCl	2 mL of 1M	4 mL of 1 M pH7.5
20 mM CaCl2	0.59 g	1.176 g

Dissolved in warmed molecular biology grade water, dispensed into 50 mL aliquots, autoclaved to sterilize.

9.3.1.3. KC-MT solution

10 mL each KC and MT. Mix in sterile universal tube just prior to use.

9.3.1.4 Protoplasting enzymes

5 mg/mL lysing enzymes (Sigma Lysing Enzymes from Trichoderma harzianum).

2 mg/mL cellulase (Sigma Cellulase from Trichoderma reesei).

Dissolve in 10 mL KC solution, filter sterilise through 0.2 µm filter. Prepare just prior to use.

9.3.1.5 PEG solution

PEG 3350	2.5 g
1 M Tris.HCl pH 7.5	50 µl
100 mM CaCl2	1.25 mL
molecular biology grade water	1.5 mL

Prepare and filter sterilise through 0.2 µm filter on day of use.

9.3.2 Yeast transformations

9.3.2.1 SORB solution

4.08 g LiAc dihydrate	4 .08g
1M Tris–HCl pH 8 (from 1 M stock)	4mL
0.5 M EDTA/NaOH pH 8	800ul
1M sorbitol	72.9g

Made up to 400mL distilled water and filter sterilised

9.3.2.2 PEG/LiAc

1 M LiAc	5mL
1M Tris–HCl pH 8	0.5Ml
0.5M EDTA/NaOH pH 8	0.1 ml

40 % PEG3350	20g

Made up to 50mL with distilled water and autoclaved for 15 mins at 121°C

9.3.2.3 TE buffer

1 M Tris-HCl (pH 8.0)	1 mL
EDTA (0.5 M)	0.2 mL

Made up to 100mL with distilled water

9.3.2.4 STET buffer

8% sucrose	6.4 g
1M Tris pH8	0.54 ml
0.5M EDTA	8 ml
5% Triton X-100	4 ml

Made up to 80 ml with distilled water and autoclaved for 15 mins at 121°C

9.4 Solutions and reagents for proteomic experiments

9.4.1 Protein extraction buffer

5% glycerol	50 ml
50 mM Tris, pH8	5 ml
100 mM NaCl	2 ml
5 mM EDTA	1 ml
2% SDS	20 ml

Made up to 100 ml with distilled water and autoclaved for 15 mins at 121°C

9.4.2 PBS buffer

Added to 800 mL of distilled water:

NaCl	8g
KCL	0.2g
Na2HPO4	1.44g
KH2PO4	0.24g

Adjusted the pH to 7.4 with HCl.

Made up to 1 L with distilled water

9.4.3 Blocking solution

Nonfat dry milk powder	7.5g
1XPBS	150ml
Tween	0.05%

9.4.4 Bead elution buffer 20 ml

Glycine	0.15g
Distilled H20	10ml

Add HCL to reduce pH to 2.5

Made up to 20 ml with distilled water and filter sterilised

9.4.5 HNT buffer 200 ml

1M HEPES pH7.4	10 ml
5M NaCl	2 ml
10% Tween-20	200 µl

Made up to 200 ml with distilled water and filter sterilised

9.4.6 HN buffer 200 ml

1M HEPES pH7.4	10 ml
5M NaCl	2 ml

Made up to 200 ml with distilled water and filter sterilised

9.4.7 Trypsin solution

10 µl of 1 µg/µL trypsin in 490 µl of 50 mM NH_4HCO_3, pH8

9.5 List of intensity values for plant proteins identified in the apoplastic fluid of infected and non-infected barley leaves at 4 and 7 dpi

Protein IDs	Non-infected 4dpi	Infected 4dpi	Non-Infected 7dpi	Infected 7dpi
MLOC_13213.1	16.0182	16.4836	16.7541	18.8455
AK367512	NaN	18.0655	NaN	19.5655
AK248469.1	NaN	17.8972	NaN	21.2479
MLOC_15778.1	NaN	17.8532	18.8101	22.2605
AK365024	NaN	21.6794	NaN	22.4553
MLOC_12105.1	NaN	18.4814	NaN	22.6346
AK357752	NaN	17.4312	NaN	22.9513
MLOC_10669.1	NaN	20.7779	NaN	23.0639
MLOC_54267.1	NaN	18.4004	NaN	23.0832
MLOC_13635.1	NaN	20.8976	NaN	23.1582
AK367422	NaN	20.3423	NaN	23.188
MLOC_16777.1	NaN	19.5908	NaN	23.3087
MLOC_63125.1	NaN	17.9103	NaN	23.8972
MLOC_68972.1	NaN	21.1732	18.6647	23.9967
AK248435.1	NaN	19.9031	NaN	24.2417
AK252734.1	NaN	19.881	NaN	24.4733
AK354347	NaN	20.4944	NaN	24.6402
MLOC_11317.1	NaN	22.4704	NaN	24.6987
MLOC_21848.2	NaN	18.8101	NaN	25.565
MLOC_52747.1	NaN	19.8281	NaN	25.8201
AK251544.1	NaN	20.4572	NaN	26.6587
MLOC_6801.1	NaN	21.1178	NaN	26.8242
AK355673	NaN	23.3581	NaN	27.313
AK360562	NaN	23.0368	NaN	28.1715
MLOC_12581.1	NaN	23.0468	NaN	28.786
MLOC_72965.1	NaN	21.4811	NaN	28.8254
AK367189	NaN	22.8901	NaN	29.3384
AK375449	NaN	19.0313	18.2276	NaN
AK248355.1	NaN	17.3614	NaN	NaN
MLOC_66864.1	NaN	17.4555	NaN	NaN
MLOC_61812.1	NaN	18.076	NaN	NaN
MLOC_75098.2	NaN	18.2285	NaN	NaN
MLOC_37207.1	NaN	18.5343	NaN	NaN
MLOC_66134.2	NaN	18.6972	NaN	NaN
AK367799	NaN	19.0479	NaN	NaN
MLOC_73077.1	31.0581	32.0898	30.9706	35.8138
MLOC_68184.1	30.2544	31.5825	30.3757	35.553
AK374484	30.1296	28.9905	27.715	31.1995
AK362756	30.0356	30.3576	28.7772	31.4367
MLOC_9957.3	29.999	30.3867	27.3175	32.0303
AK248896.1	29.8551	30.7494	30.8831	34.2715
AK372381	29.5315	27.7762	28.5175	29.1053
MLOC_65311.2	29.5105	31.0484	30.0603	34.9329
AK364106	29.4221	27.4497	26.3037	27.8948
AK252303.1	29.2943	30.314	27.5979	33.4395
MLOC_64136.1	29.2362	28.4083	25.5978	29.3984
MLOC_56418.3	28.8636	27.8249	26.346	29.538
AK251990.1	28.5232	30.3295	28.675	34.523
AK357344	28.4284	28.5663	27.545	31.3299
MLOC_26558.1	28.4134	28.5502	25.7379	34.1756
AK361831	28.4084	27.9094	27.4109	29.5713
MLOC_75626.	28.0301	27.421	23.915	28.9566
MLOC_78725.2	27.4894	27.9205	26.2261	30.6011

Protein IDs	Non-infected 4dpi	Infected 4dpi	Non-Infected 7dpi	Infected 7dpi
AK252669.1	27.368	27.922	24.6631	30.7916
AK358031	27.3223	26.6608	24.8374	28.4323
MLOC_59790.1	27.3157	26.7213	18.8088	28.0939
MLOC_10319.1	27.1358	26.2993	21.1226	25.9783
AK363456	27.0807	26.0367	23.2226	27.4336
AK361492	27.0646	28.1747	25.2466	32.3512
MLOC_37675.1	26.924	26.5802	21.126	28.222
MLOC_62475.1	26.8521	24.6738	NaN	24.8554
MLOC_10769.1	26.8138	26.6203	18.1591	29.5023
MLOC_55663.1	26.7301	29.0279	27.1079	33.5072
MLOC_65225.2	26.6305	29.1304	27.3526	33.7739
AK370002	26.6288	28.7466	27.5947	33.4087
AK355059	26.4313	28.8902	25.128	34.297
AK367302	26.368	26.6636	25.6652	26.1649
AK354993	26.3354	27.8506	25.7185	29.0586
AK365289	26.2105	25.0178	16.6548	24.0302
MLOC_81871.1	26.1759	26.5572	22.8087	29.07
MLOC_69589.1	26.1249	29.2317	25.6148	33.7407
AK370783	26.0914	27.0166	23.4616	30.3286
AK363219	26.0822	25.8385	NaN	30.717
AK366716	26.0252	26.4129	23.539	32.6462
MLOC_58156.1	25.9895	24.6095	22.5233	25.6913
MLOC_44256.2	25.9213	25.0889	23.2949	25.9366
MLOC_60721.1	25.8373	22.1371	24.7568	25.8422
MLOC_59521.2	25.7017	23.6378	NaN	25.0188
AK362004	25.6904	24.9304	24.0211	26.5308
AK356825	25.6575	20.9905	19.1454	21.464
MLOC_63550.2	25.6448	25.7088	23.3188	28.3063
MLOC_15203.1	25.6398	23.87	20.8275	23.1823
MLOC_55142.1	25.5098	23.341	23.5584	23.7373
MLOC_62056.2	25.4793	24.656	20.5232	NaN
MLOC_61558.1	25.4149	24.0058	26.3504	29.354
MLOC_67715.1	25.084	25.0984	NaN	28.4921
MLOC_71887.2	24.8801	25.592	21.7643	28.6359
AK357890	24.8177	23.4176	21.0201	22.1902
MLOC_12359.2	24.6062	23.8243	18.9405	24.6828
MLOC_54205.1	24.4838	19.7044	20.6985	21.7036
AK356944	24.4233	25.611	20.8105	26.2001
MLOC_72498.1	24.3439	27.4606	22.0448	31.51
AK354002	24.322	25.1894	19.9454	26.7236
MLOC_32914.2	24.3075	25.0571	23.5915	25.9412
MLOC_62746.1	24.2884	25.9885	23.594	29.8582
AK249082.1	24.2513	21.3701	23.4762	29.2246
AK358571	24.1253	24.9599	NaN	28.4283
MLOC_71858.2	24.0998	25.0109	23.7961	28.8638
MLOC_39318.1	24.0258	26.8983	22.4426	31.5309
MLOC_71570.1	23.979	22.0441	16.9996	NaN

Protein IDs	Non-infected 4dpi	Infected 4dpi	Non-Infected 7dpi	Infected 7dpi
MLOC_12288.1	23.9025	21.1749	20.7683	NaN
MLOC_60157.3	23.8924	24.5643	20.4555	18.9857
MLOC_71242.1	23.7309	26.6263	NaN	31.1793
MLOC_44817.1	23.7206	25.9858	23.3629	29.8207
MLOC_72826.1	23.716	24.204	NaN	27.7264
AK358923	23.7128	24.4619	22.6593	26.0136
AK371130	23.7023	25.6349	NaN	28.123
AK249257.1	23.6764	23.4721	20.0152	23.9628
AK369753	23.6577	23.3928	21.5317	NaN
AK373131	23.6202	25.5756	23.0937	29.6166
MLOC_13908.1	23.5153	26.9958	25.2675	30.3252
MLOC_72727.1	23.5128	23.7133	19.5621	27.0163
MLOC_52040.2	23.4958	23.9696	21.6628	26.4234
AK365716	23.4873	23.152	NaN	NaN
MLOC_62622.2	23.4644	26.004	23.9321	29.3484
AK364296	23.416	25.2493	24.461	28.4377
AK363288	23.3543	21.5206	NaN	22.7393
MLOC_72157.3	23.2999	24.7578	NaN	31.5154
MLOC_3334.1	23.2707	23.5536	NaN	NaN
AK367409	23.2459	22.3223	21.5967	23.5015
MLOC_56250.1	23.2333	NaN	NaN	23.3475
MLOC_19686.5	23.2089	24.5046	23.9649	24.715
MLOC_55542.1	23.1769	21.1918	NaN	25.2119
MLOC_64967.1	23.1756	22.9008	NaN	26.7407
MLOC_1587.2	23.1263	25.203	NaN	27.8739
MLOC_62394.2	23.1142	26.2367	21.9513	31.5248
MLOC_58958.2	23.0854	23.1189	22.6108	26.169
AK359422	23.0782	NaN	NaN	NaN
MLOC_69905.3	23.0778	23.5129	20.2195	24.2448
AK252245.1	23.074	19.0862	20.8126	25.0413
MLOC_44443.1	23.0021	23.9265	NaN	30.3794
MLOC_57757.1	22.9428	24.6626	22.4202	28.0671
AK253091.1	22.9101	NaN	19.462	23.2711
AK371884	22.9044	23.9709	22.496	NaN
AK354832	22.8804	22.461	NaN	25.8705
MLOC_75385.1	22.8591	NaN	NaN	NaN
MLOC_12220.1	22.762	NaN	NaN	19.6907
AK252358.1	22.761	22.2899	20.9267	24.756
MLOC_50007.1	22.7299	21.7832	20.7713	20.4746
MLOC_65477.1	22.6887	24.9903	NaN	28.4236
AK361477	22.6825	23.4333	22.3551	28.3813
MLOC_10457.2	22.6707	22.6092	20.2985	25.0968
MLOC_80476.1	22.6688	23.7881	17.7579	28.9822
AK354860	22.6025	NaN	NaN	21.0778
MLOC_17554.1	22.4925	24.1952	NaN	25.9862
MLOC_39127.1	22.4737	25.2043	20.8793	30.7134
MLOC_77721.1	22.3923	22.2913	NaN	28.235
MLOC_65883.1	22.3505	18.3062	NaN	23.3932
MLOC_23312.1	22.266	22.8671	21.0364	NaN
MLOC_11960.2	22.2218	21.7215	NaN	24.9055
MLOC_57254.8	22.077	21.4667	20.0391	22.3991
MLOC_12179.1	21.991	19.6843	NaN	24.7831
MLOC_36459.1	21.9729	19.9304	NaN	27.8644
MLOC_77485.3	21.9714	NaN	18.5715	18.9335

Protein IDs	Non-infected 4dpi	Infected 4dpi	Non-Infected 7dpi	Infected 7dpi
MLOC_58866.1	21.9634	NaN	NaN	NaN
MLOC_47346.1	21.9626	23.6678	20.7214	27.9334
MLOC_79567.1	21.9013	25.65	18.4087	28.6326
AK359722	21.8296	18.9731	NaN	19.9836
MLOC_73299.1	21.827	NaN	NaN	20.505
AK370843	21.823	23.0537	NaN	26.9707
AK365832	21.7731	20.7894	NaN	20.0842
AK354935	21.7474	NaN	17.4271	NaN
AK368976	21.7438	18.6252	NaN	NaN
AK358814	21.7215	20.7802	NaN	25.2637
AK374179	21.6094	24.472	NaN	25.7451
MLOC_58454.1	21.5911	19.9565	NaN	NaN
MLOC_17055.1	21.5856	20.4002	NaN	22.1271
AK358941	21.5644	20.235	NaN	21.4548
MLOC_18287.4	21.4472	16.915	20.0414	19.4386
MLOC_65226.3	21.4184	22.9318	NaN	30.548
MLOC_13009.1	21.3884	19.3787	19.3285	21.5545
AK356701	21.3444	NaN	NaN	NaN
MLOC_74370.1	21.2663	23.5643	NaN	28.101
MLOC_58946.2	21.2425	19.5562	NaN	23.8134
AK366148	21.2081	20.5851	17.4741	21.6585
AK35760	21.1902	20.6493	18.6121	NaN
AK248526.1	21.1598	23.1696	NaN	21.0342
AK361150	21.1061	23.7137	NaN	26.2866
AK362964	21.0718	NaN	NaN	NaN
MLOC_43331.1	21.0297	NaN	24.0977	26.2292
MLOC_74354.1	20.9899	NaN	NaN	NaN
MLOC_61193.1	20.9526	NaN	NaN	NaN
MLOC_80849.1	20.9001	21.4774	NaN	NaN
MLOC_5168.1	20.876	23.9452	19.5165	26.3529
MLOC_78379.1	20.8596	20.9391	NaN	NaN
AK369109	20.8244	19.3849	NaN	18.5331
MLOC_60447.1	20.6872	20.6596	NaN	22.5342
MLOC_61064.1	20.6508	NaN	NaN	NaN
MLOC_73157.2	20.6299	20.6096	NaN	NaN
MLOC_4511.1	20.5609	23.0764	NaN	24.814
AK359384	20.5274	NaN	NaN	NaN
MLOC_72199.1	20.5221	21.2577	20.912	23.1207
AK361047	20.474	19.4717	NaN	NaN
AK353926	20.4232	19.7512	NaN	24.9276
MLOC_58795.3	20.4075	20.8263	NaN	NaN
MLOC_7366.1	20.4035	22.5571	NaN	27.8423
MLOC_20045.2	20.3466	21.7957	NaN	23.1604
AK364039	20.3297	22.508	NaN	21.3632
MLOC_78015.1	20.2038	17.8336	NaN	NaN
MLOC_70480.1	20.0671	20.7535	NaN	NaN
AK360353;	20.0596	18.8047	NaN	NaN
AK369798	20.0196	19.6432	21.4645	NaN
AK362010;	20.0103	20.1084	NaN	NaN
MLOC_6896.2	19.9086	NaN	NaN	NaN
MLOC_74142.1	19.876	NaN	NaN	21.8178
MLOC_4840.1	19.8545	18.8071	NaN	22.7022
MLOC_80571.3	19.8539	19.7472	NaN	NaN
AK369156	19.7686	17.8166	NaN	NaN

Protein IDs	Non-infected 4dpi	Infected 4dpi	Non-Infected 7dpi	Infected 7dpi
MLOC_77701.1	19.7562	NaN	NaN	24.0342
MLOC_19306.1;	19.6518	NaN	NaN	NaN
AK360979	19.6353	21.5407	19.1842	24.1684
MLOC_44817.1	23.7206	25.9858	23.3629	29.8207
MLOC_14380.1;	19.5907	19.5906	NaN	NaN
MLOC_13056.2	19.5637	20.312	NaN	24.4729
AK364955	19.4497	NaN	NaN	19.0224
AK353768	19.3929	18.9616	NaN	NaN
AK360100	19.3694	21.837	NaN	22.4805
AK360188	19.3654	18.8138	NaN	20.732
AK375045	19.3177	21.1325	20.4383	23.7734
AK358939	19.234	19.0051	NaN	19.487
AK251422.1	19.2102	23.0238	18.0205	31.0514
MLOC_43759.1	19.207	19.7845	NaN	26.8019
MLOC_59924.2	19.2063	NaN	20.2464	23.4125
MLOC_75889.3	19.2001	18.6285	NaN	NaN
MLOC_78015.1	20.2038	17.8336	NaN	NaN
MLOC_72826.1	23.716	24.204	NaN	27.7264
MLOC_71275.2	19.1524	NaN	NaN	NaN
MLOC_73713.1	19.1113	19.185	NaN	NaN
AK357127	19.1011	17.8016	NaN	18.8709
MLOC_76000.8	19.0197	NaN	NaN	NaN
MLOC_10261.1	19.0152	NaN	NaN	NaN
AK250129.1	18.9907	NaN	NaN	NaN
AK362698	18.9703	NaN	NaN	19.1994
MLOC_53738.2	18.9685	18.7347	NaN	24.2394
MLOC_21159.1	18.9256	NaN	NaN	NaN
AK355811	18.8935	21.4053	NaN	25.2018
MLOC_62162.1	18.7761	20.6494	NaN	NaN
MLOC_73268.1	18.7718	18.4175	NaN	NaN
AK365489	18.7043	18.7277	NaN	23.165
AK250154.1	18.5197	NaN	NaN	NaN
MLOC_15761.1	18.4078	NaN	NaN	18.9551
MLOC_9865.1	18.3315	17.6955	NaN	21.4998
MLOC_39273.1	18.3079	NaN	NaN	NaN
MLOC_4597.1	18.3035	18.5964	NaN	NaN
MLOC_72162.1	18.3022	19.4707	17.7137	NaN
AK249308.1	18.2253	NaN	NaN	NaN
MLOC_64782.2	18.1797	NaN	NaN	19.1648
MLOC_34836.3	18.1021	NaN	NaN	NaN
AK356601	18.0919	18.9071	NaN	NaN
MLOC_71416.1	17.962	19.8607	18.07	24.2463
MLOC_57195.1	17.9122	NaN	NaN	21.2282
MLOC_79176.1	17.7201	20.7786	NaN	28.1525
AK373354	17.6371	NaN	NaN	23.4975
MLOC_34608.2	17.4744	NaN	NaN	NaN
MLOC_20723.1	17.3146	20.6511	NaN	25.2754
AK374035	17.1737	18.995	NaN	23.1298
AK355770	16.915	NaN	NaN	20.2289
MLOC_69708.1	16.8155	22.3502	NaN	27.7559
AK363344	16.3355	15.3245	NaN	20.1772
AK372814	16.0379	NaN	NaN	NaN
AK248864.1	NaN	NaN	16.6243	NaN

9.6 List of proteins identified in the apoplastic fluid from cultivars Optic, Atlas and Atlas46, infected and non-infected leaf material

protein Id	Signal P	Target P	Best BLAST hit	e-value	accesssion #
AK357003	-	other	putative S-adenosylhomocystein hydrolase 2 [Hordeum vulgare]	0	CAJ01707.1
AK357872	-	other	predicted protein [Hordeum vulgare subsp. vulgare]	0	BAJ89086.1
AK364106	-	other	Lysosomal alpha-mannosidase [Aegilops tauschii]	0	EMT01827.1
AK374484	25/26	SP	beta-D-glucan exohydrolase isoenzyme ExoI [Hordeum vulgare]	0	AAD23382.1
MLOC_80476.1	24/25	SP	wali6 [Triticum aestivum]	1E-53	AAC37417.1
AK354891	24/25	SP	Subtilisin-like protease [Triticum urartu]	0	EMS48667.1
AK376814	-	other	Triosephosphate isomerase, chloroplastic [Aegilops tauschii]	3E - 90	EMT18057.1
MLOC_74692.1	-	other	hypothetical protein F775_06906 [Aegilops tauschii]	1E-38	EMT28254.1
AK374192	-	other	universal stress protein MT2085 [B. distachyon]	5E-87	XP_003562944.1
MLOC_72417.2	-	other	differentially expressed in FDCP 6 homolog [B. distachyon]	0	XP_003561472.1
MLOC_74692.1	-	other	hypothetical protein F775_06906 [Aegilops tauschii]	1-E38	EMT28254.1
AK376379	-	other	ankyrin repeat and KH domain-containing protein 1 [B. distachyon]	0	XP_003580025.1
MLOC_52687.1	-	other	2,3-bisphosphoglycerate-independent phosphoglycerate mutase [Brachypodium distachyon]	0	XP_003564482.1
MLOC_60721.1	22/23	SP	probable beta-D-xylosidase 6 [Brachypodium distachyon]	0	XP_003580200.2
MLOC_12553.1	-	other	hypothetical protein TRIUR3_21222 [Triticum urartu]	3E-101	EMS54120.1
MLOC_39605.1	-	other	Protein NEDD1 [Triticum urartu]	3E-82	EMS68148.1
AK356611	-	other	polyadenylate-binding protein RBP45-like [B. distachyon]	4E-137	XP_003573391.1
MLOC_18832.1	27/28	SP	PREDICTED: cationic amino acid transporter 5 [B. distachyon]	0	XP_003565484.1
MLOC_38006.2	-	other	PREDICTED: glycine-rich protein 1 isoform X2 [B. distachyon]	9E-49	XP_014752896.1
MLOC_43545.1	-	other	Putative disease resistance protein RGA4 [Triticum urartu]	0	EMS58751.1
AK357344	-	other	Glucan 1,3-beta-glucosidase [Aegilops tauschii]	0	EMT07888.1
AK370199	-	other	Disease resistance RPP13-like protein 4 [Triticum urartu]	0	EMS56997.1
AK370340	-	MTP	expressed protein 1 [Triticum aestivum]	0	ACU21593.1
MLOC_16741.1	-	other	hypothetical protein TRIUR3_34254 [Triticum urartu]	0	EMS57010.1
MLOC_63550.2	25/26	SP	Reticuline oxidase-like protein [Aegilops tauschii]	0	EMT13084.1